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# A phylogenetic analysis of conifer diterpenoids and their carbon isotopes for chemotaxonomic applications

Aaron F. Diefendorf [a,*], Andrew B. Leslie [b], Scott L. Wing [c]

[a] Department of Geology, University of Cincinnati, Cincinnati, OH 45221, USA
[b] Department of Ecology and Evolutionary Biology, Brown University, Providence, RI 02912, USA
[c] Department of Paleobiology, Smithsonian Institution, Washington, DC 20013, USA

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ABSTRACT

Plant-derived diterpenoids are commonly used as conifer-specific biomarkers and for chemotaxonomic assignment or confirmation. Numerous studies have reported on the utility of diterpenoids as chemotaxonomic indicators, but few have quantitatively analyzed diterpenoid concentrations, especially with respect to conifer phylogeny. In addition, the carbon isotope values ($\delta^{13}C$) of diterpenoids provide a means to track conifer-specific $\delta^{13}C$ values, which is useful for tracking water availability and the carbon cycle. To expand on previous work, we measured diterpenoid concentrations and carbon isotopes of 43 conifer species, and *Ginkgo biloba*, collected at the University of California Botanical Garden at Berkeley. In this collection, all extant conifer families and almost two-thirds of extant genera are present, including many subtropical and Southern Hemisphere groups that were once common and widespread components of fossil assemblages. Overall, we found diterpenoid concentrations were highly variable among species and families. Despite this variability, there is coarse family-level phylogenetic structuring with the lowest concentrations in Pinaceae. When diterpenoid concentrations are fitted to a model of trait evolution (Brownian motion), we however find that there is no phylogenetic signal. In contrast, when terpenoids are analyzed by the proportion of diterpenoid compound structure classes (abietanes, labdanes, pimaranes, tetracyclics, and totarols/sempervirol), there was significant phylogenic signal for the abietane and tetracyclic structures. Diterpenoid biosynthetic carbon isotope fractionation, as expressed between diterpenoids and leaf tissue ($\varepsilon_{diterpenoid}$), also contained a phylogenetic signal, as well as the broad phylogenetic structuring observed in total diterpenoid concentrations. Overall, these results indicate that the Pinaceae is unique among conifer clades with respect to terpenoid structure classes, concentrations and $\varepsilon_{diterpenoid}$. When diterpenoids are applied to taxonomic assignment of fossils, it would be useful to combine several traits (concentration, proportion, and $\varepsilon_{diterpenoid}$). In this context, it should be possible to broadly distinguish three major conifer groups: Cupressaceae, Podocarpaceae and Pinaceae. However, based on these results, we recommend against assuming that closely related species have similar diterpenoid compositions.



## 1. Introduction

Plant terpenoids represent one of the largest and most diverse groups of vascular plant lipids (e.g., Tholl, 2015). The function of these compounds is diverse and includes chemical defense against insect and pathogen attack, as well as other functions (Langenheim, 1994; Chaturvedi et al., 2012; Kemen et al., 2014; Tholl, 2015). Plant terpenoids are of great interest to organic geochemists because they can provide plant taxonomic specificity. For example, many diterpenoids such as the abietane, beyerane, kaurane, labdane, phyllocladane, pimarane, and totarane structure classes (Fig. 1) are unique to gymnosperm clades such as conifers and *Ginkgo* (Erdtman, 1963; Dev, 1989; Otto et al., 1997; Otto and Simoneit, 2001; Otto and Wilde, 2001; Hautevelle et al., 2006; Keeling and Bohlmann, 2006; Cox et al., 2007). In contrast, many non-steroidal pentacyclic triterpenoids are specific to angiosperms (ten Haven and Rullkötter, 1988; Woolhouse et al., 1992; Otto and Simoneit, 2001; Diefendorf et al., 2012). Variations in the concentrations of terpenoids, especially di- and triterpenoids, have been used to identify changes in floral composition (Pancost et al., 2002; Bechtel et al., 2003, 2005; Pancost and Boot, 2004; Grice et al., 2005; Hautevelle et al., 2006; Schouten et al., 2007) and to identify the rise of angiosperms and their ancestors (Moldowan et al., 1994).

* Corresponding author.
*E-mail address:* aaron.diefendorf@uc.edu (A.F. Diefendorf).

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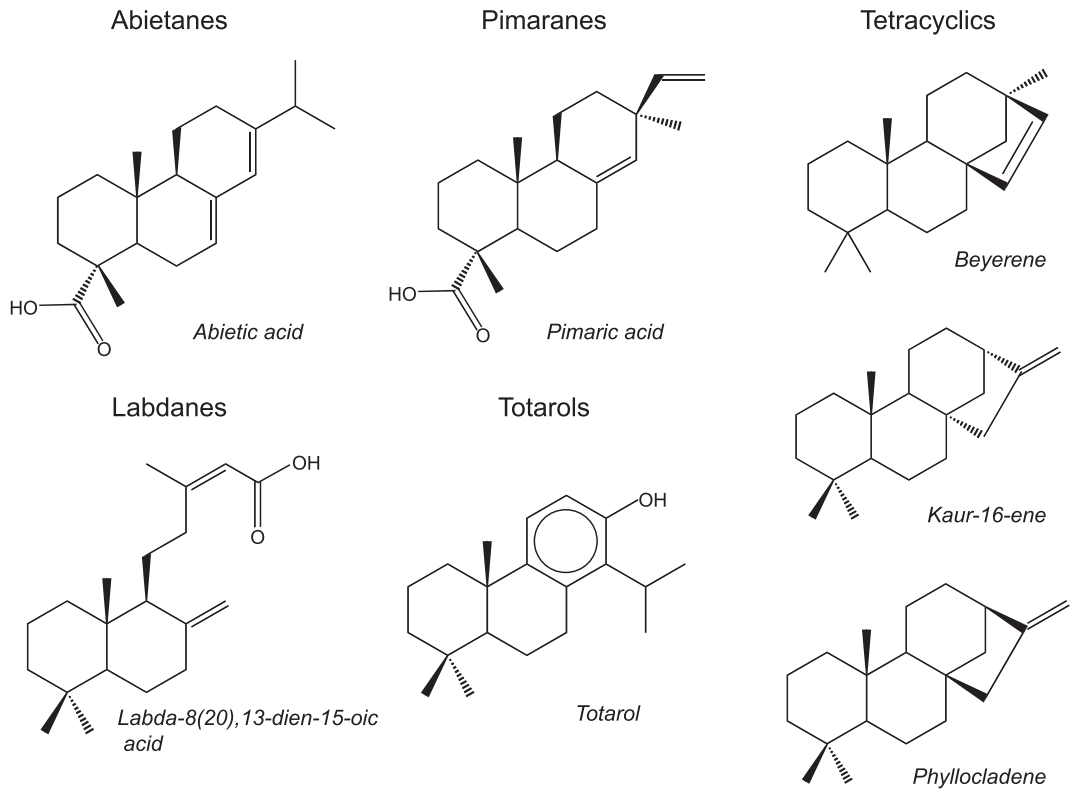


**Fig. 1.** Examples of common diterpenoid compounds of each of the major compound class structures found in many of the species analyzed.

Within gymnosperms, conifers are the most species-rich extant group and have the most abundant and diverse fossil record, stretching back more than 300 million years (e.g., Taylor et al., 2009). Any potential variation in diterpenoid biochemistry among conifer clades could thus be very valuable in attempting to determine which specific groups were abundant at different time periods, which is important in reconstructing ancient community structure and paleoecology. The use of diterpenoids as chemotaxonomic indicators for the assignment of conifer fossils to families and genera is common (Otto and Simoneit, 2002; Otto et al., 2003, 2005) and is based on many exhaustive studies of extant conifers and comparisons of closely related extant and extinct species (e.g., Otto and Wilde, 2001; Cox et al., 2007; Simoneit et al., 2016). However, at least in general, diterpenoid compound structures have low specificity to the broader conifer families (Otto and Wilde, 2001; Hautevelle et al., 2006). This potentially limits the utility of chemotaxonomy for fossils that lack close living relatives.

In order to test if there is any deeper pattern to diterpenoid composition across the major conifer groups that might be useful when interpreting the fossil biomarker record, we provide here a comparison of diterpenoid concentrations and carbon isotope values ($\delta^{13}C$), as well as associated biosynthetic fractionation factors ($\varepsilon_{diterpenoid}$), across a representative sample of living conifers and *Ginkgo*. Our goal was to compare diterpenoid abundance and composition in representative members of all six major extant conifer clades (Rai et al., 2008; Leslie et al., 2012) and test if there is any overarching phylogenetic structure or signal in these data.

## 2. Materials and methods

### 2.1. Samples

A detailed description of the samples, extraction, separation, and analysis can be found in Diefendorf et al. (2015) where the *n*-alkyl lipids were reported from the same set of samples. In brief, fresh leaf tissue was collected from 43 conifer species and from *Ginkgo biloba* at the University of California Botanical Garden at Berkeley (UCBGB; 37.8752°N, 122.2386°W, 210 m) in December 2011. For each species, leaf tissue was collected from multiple branches on the sun-exposed side of a single mature tree per species with sampling height of 2–3 m, with the exception of *Sequoiadendron giganteum* and *Sequoia sempervirens*, which were sampled at ~25 m. The canopy structure at the UCBGB is very open and, therefore, it is unlikely that soil respiration generates a $^{13}C$-depleted $CO_2$ layer that could have influenced leaf and terpenoid $\delta^{13}C$ values.

### 2.2. Extraction and fractionation

Powdered leaves were extracted using an accelerated solvent extractor (Dionex ASE 350) with 2:1 (v/v) DCM/MeOH. The total lipid extract (TLE) was base saponified to cleave ester groups with 2.5 ml 0.5 N KOH in 3:1 (v/v) MeOH/water for 2 h at 75 °C. After cooling, 2 ml of NaCl in water (5%, w/w) was added and then the solution was acidified with 6 N HCl to a pH of 1. The acidic solution was extracted with hexanes/DCM (4:1, v/v), neutralized with $NaHCO_3/H_2O$ (5%, w/w), and dried with $Na_2SO_4$. The TLE was separated into four polarity fractions by elution through 0.5 g aminopropyl-bonded silica gel. Hydrocarbons were eluted with 4 ml of hexanes, ketones eluted with 8 ml of hexanes/DCM (6:1, v/v), alcohols eluted with 8 ml of DCM/acetone (9:1, v/v), and acids eluted with 8 ml of DCM/formic acid (49:1, v/v).

### 2.3. Lipid assignment and quantification

Lipids were identified and quantified on an Agilent 7890A gas chromatograph (GC) interfaced to an Agilent 5975C quadrupole mass selective detector (MSD; operated at 70 eV) and flame ioniza-

tion detector (FID). Compounds were separated on a fused silica capillary column (Agilent J&W DB-5 ms; 30 m length, 0.25 mm i.d., 0.25 μm film thickness). For hydrocarbons, the oven program was as follows: 60 °C for 1 min, followed by a ramp (6 °C/min) to 320 °C and held for 15 min. For all other fractions, the oven program was as follows: 60 °C for 1 min, followed by a ramp (20 °C/min) to 130 °C, then a ramp (4 °C/min) to 320 °C and held for 10 min. Following GC separation, the column effluent was split (1:1) between the FID and MSD with a 2-way splitter with He makeup to keep pressure constant. GC conditions were the same as those reported in Diefendorf et al. (2015). Compounds were identified with authentic standards, library databases (NIST 2008 and Wiley 2009), published spectra (Philp, 1985; Adams, 2001; Otto and Simoneit, 2001; Otto et al., 2002; Latorre et al., 2003; Stacey et al., 2006; Cox et al., 2007; Simoneit et al., 2016), spectral interpretation, and by retention time (Table 1).

For quantification by FID, hydrocarbons were dissolved quantitatively in hexanes spiked with 10 μg/ml 1,1′-binapthyl as the internal standard. For the alcohol and acid fractions, aliquots of the saponified lipid extract were dissolved in pyridine spiked with 20 μg/ml of 2-dodecanol and derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA; Sigma Aldrich). Compound peak areas were normalized to the internal standard and converted to mass with external standard response curves, also normalized to the internal standards, ranging in concentration from 0.5 to 100 μg/ml. External standards included $C_7$ to $C_{40}$ *n*-alkanes, *n*-alkanols ($C_{16}$, $C_{18}$, $C_{22}$, $C_{26}$, $C_{28}$), *n*-alkanoic acids ($C_{10}$, $C_{14}$, $C_{16}$, $C_{18}$, $C_{24}$, $C_{26}$, $C_{28}$, $C_{30}$), and isopimaric acid. Compound concentrations were normalized to the dry leaf mass (μg/g).

### 2.4. Carbon isotope analysis

Compound-specific carbon isotope analyses of the diterpanes and diterpenes were determined by GC–combustion (C)-IRMS. Prior to analysis, hydrocarbons were separated into saturated and unsaturated fractions, with 4 ml of hexanes and 4 ml of ethyl acetate, respectively, on 0.5 g of 5% $Ag^+$-impregnated silica gel (w/w). GC–C-IRMS was performed with a Thermo Trace GC Ultra coupled to an Isolink combustion reactor (Ni, Cu, and Pt) and Thermo Electron Delta V Advantage IRMS. Isotopic abundances were determined relative to a reference gas calibrated with Mix A6 (Arndt Schimmelmann, Indiana University). Carbon isotope accuracy was monitored with co-injected *n*-$C_{41}$ alkane and the precision and accuracy were 0.23‰ (1σ; n = 61) and −0.13‰, respectively, over the course of analysis. Leaf and *n*-alkane $\delta^{13}C$ values were measured and reported in Diefendorf et al. (2015).

Fractionation that occurs during diterpenoid biosynthesis was calculated relative to bulk leaf tissue ($\delta^{13}C_{leaf}$) using epsilon notation where:

$$\varepsilon_{diterpenoid} = \left(\frac{\delta^{13}C_{diterpenoid}+1}{\delta^{13}C_{leaf}+1} - 1\right) \quad (1)$$

A concentration-weighted $\varepsilon_{diterpenoid}$ value ($\varepsilon_{diterp\text{-}weighted}$) was determined by weighting each diterpenoid value by its concentration.

### 2.5. Phylogenetic analysis and statistics

To evaluate the effect of evolutionary relationships on diterpenoids, we grouped conifer species according to major clades, as recognized by previous phylogenetic studies (Rai et al., 2008; Leslie et al., 2012). These include the traditionally recognized conifer families including the Araucariaceae, Cupressaceae, Pinaceae, Podocarpaceae, Sciadopityaceae, and Taxaceae. We also analyzed three additional subclades of Cupressaceae including the Cupressoideae (Northern Hemisphere cypresses and junipers), the Callitroideae (Southern Hemisphere cedars), and the taxodioid Cupressaceae, an informal group of early-diverging lineages that includes species such as redwoods and bald cypresses that are abundant in the fossil record.

All phylogenetic analyses in this study used a time-calibrated molecular conifer phylogeny from Leslie et al. (2012). This tree was subsampled to include only the 43 conifer species used in this analysis. *Ginkgo* was not included in the Leslie et al. (2012) study and was therefore excluded from the phylogenetic analyses. The phylogenetically adjusted PCA and phylogenetic signal tests were performed using the package phytools (Revell, 2012) in the open source statistical software R version 3.1.2 (R core team, 2014). Other statistical analyses were preformed using R and JMP Pro 12.0 (SAS, Cary, USA).

In the context of the phylogenetic grouping, we used a principal components analysis (PCA) of the total concentrations of diterpenoid compound structure classes for all species to identify any basic patterns in the data. We also repeated this PCA using the proportions of each of the compound structure classes. For these analyses, we used a phylogenetically corrected PCA, a statistical correction which accounts for the phylogenetic non-independence of the data (Revell, 2009). This approach uses a phylogenetic tree and a Brownian motion (BM) model of trait evolution to calculate expected trait covariance due to relatedness. With this method, closely related species should have similar trait values while more distantly related taxa should have more divergent values. This phylogenetic covariance matrix is then used in the PCA analysis of the correlation matrix of trait values. In essence, the analysis uses the non-phylogenetic residual variation among the taxa to compute eigenvalues and eigenvectors, so that taxa are more appropriately ordinated on principal component axes (Revell, 2009). Studies suggest this method can reduce error in statistical results, even if the data are subsequently used in further phylogenetic analyses (see Revell, 2009).

We then tested for a phylogenetic signal in each of our trait variables (total diterpenoid concentration, concentrations of each compound structure, proportion of compound structure, and $\varepsilon_{diterpenoid}$ values), Principal Components Axis 1 (PC 1) scores of total diterpenoid concentrations, and PC 1 scores for the proportion of each structure class, using the *K*-statistic of Blomberg et al. (2003) and Pagel's λ (Pagel, 1999). These statistics are different ways to measure how well the distribution of observed trait values follows expectations from a BM process given the phylogenetic relationships among the species, where a value of 1.0 would indicate a perfect correlation with expectations.

## 3. Results and discussion

### 3.1. Diterpenoid concentrations

Diterpenoids were present in all species. However, diterpenoid composition and concentration were highly variable among species and families with total diterpenoid concentrations spanning four orders of magnitude (1–16,900 μg/g; Fig. 2; Table 2 and Supplementary Table S1). Abietanes were present in all species, but also with high variability. The pimaranes were common but were not found in *G. biloba* and *Cathaya argyrophylla*. The labdanes were detected in most species (82%) with the exception of many species in the Pinaceae family and *G. biloba*. We did not detect labdanes (such as isocupressic acid) in Pinaceae species studied here, though they are known to be abundant in other pine species, such as *Pinus ponderosa*, *P. jeffreyi*, *P. contorta*, and a few *Juniperus* species (Gardner and James, 1999). The tetracyclic diterpenoids (e.g., beyerane, kaurane, phyllocladane), totarols and sempervirol were

**Table 1**
Diterpenoid compounds quantified in this study along with their retention index, composition, molecular weight (MW), and the method of compound assignment.

| Name | Kovats retention index | Composition[a] | MW[a] | ID[b] | References |
|---|---|---|---|---|---|
| **Abietanes** | | | | | |
| Abietatriene (Abieta-8,11,13-triene) | 2065 | $C_{20}H_{30}$ | 270 | P | Adams et al., 2001 |
| Abietadiene (Abieta-7,13-diene) | 2099 | $C_{20}H_{32}$ | 272 | P | Adams et al., 2001 |
| Abieta-8(14),13(15)-diene (neo-Abietadiene) | 2161 | $C_{20}H_{32}$ | 272 | P | Adams et al., 2001 |
| Ferruginol methyl ether | 2213 | $C_{21}H_{32}O$ | 290 | L | |
| 2,3-Dehydroferruginol | 2225 | $C_{23}H_{36}OSi$ | 356 | P | Cox et al., 2007 |
| Ferruginol | 2232 | $C_{23}H_{38}OSi$ | 358 | P | Cox et al., 2007; Otto and Simoneit, 2001 |
| 6,7-Dehydroferruginol | 2243 | $C_{23}H_{36}OSi$ | 356 | P | Otto and Simoneit, 2001 |
| Dehydroabietol | 2293 | $C_{23}H_{38}OSi$ | 358 | P | Otto and Simoneit, 2001 |
| Pisiferol (20-Hydroxyerruginol) | 2312 | $C_{26}H_{46}O_2Si_2$ | 446 | P | Otto and Simoneit, 2001; Simoneit et al., 2016 |
| Dehydroabietic acid | 2373 | $C_{23}H_{36}O_2Si$ | 372 | L | |
| *abeo*-Pisiferol | 2378 | $C_{23}H_{38}O_2Si$ | 374 | P | Simoneit et al., 2016 |
| Hinokiol | 2389 | $C_{26}H_{46}O_2Si_2$ | 446 | P | Simoneit et al., 2016 |
| Abietic acid | 2409 | $C_{23}H_{38}O_2Si$ | 374 | L | |
| 3-Hydroxyferruginol | 2482 | $C_{26}H_{46}O_2Si_2$ | 446 | P | Cox et al., 2007 |
| 2-Hydroxyferruginol | 2491 | $C_{26}H_{46}O_2Si_2$ | 446 | P | Cox et al., 2007 |
| Sugiol | 2507 | $C_{23}H_{36}O_2Si$ | 372 | P | Otto et al., 2002; Simoneit et al., 2016 |
| **Pimaranes** | | | | | |
| Rimuene (13S-Rosa-5,15-diene) | 1910 | $C_{20}H_{32}$ | 272 | P | Adams et al., 2001 |
| Isopimara-9(11),15-diene | 1917 | $C_{20}H_{32}$ | 272 | P | Adams et al., 2001 |
| Rosa-5,15-diene, <ent-> | 1943 | $C_{20}H_{32}$ | 272 | P | Adams et al., 2001 |
| Pimaradiene (Pimara-8(14),15-diene) | 1958 | $C_{20}H_{32}$ | 272 | P | Adams et al., 2001 |
| Sandaracopimara-8(14),15-diene | 1977 | $C_{20}H_{32}$ | 272 | P | Adams et al., 2001 |
| Isopimara-7,15-diene | 2011 | $C_{20}H_{32}$ | 272 | L | |
| Dolabradiene, <13-epi> | 2020 | $C_{20}H_{32}$ | 272 | P | Adams et al., 2001 |
| Nezukol | 2151 | $C_{20}H_{34}O$ | 290 | P, L | Adams et al., 2001 |
| Pimaric acid | 2290 | $C_{23}H_{38}O_2Si$ | 374 | L | |
| Sandaracopimaric acid isomer | 2295 | $C_{23}H_{38}O_2Si$ | 374 | I | |
| Isopimaric acid isomer | 2305 | $C_{23}H_{38}O_2Si$ | 374 | I | |
| Sandaracopimaric acid | 2308 | $C_{23}H_{38}O_2Si$ | 374 | P | Stacey et al., 2006; Latorre et al., 2003 |
| Isopimaric acid | 2326 | $C_{23}H_{38}O_2Si$ | 374 | L, S | |
| **Labdanes** | | | | | |
| Sclarene | 1990 | $C_{20}H_{32}$ | 272 | P | Adams et al., 2001 |
| Manoyl oxide | 2005 | $C_{20}H_{34}O$ | 290 | P | Adams et al., 2001 |
| Labdanoic acid unknown #1 | 2233 | $C_{23}H_{38}O_2Si$ | 374 | I | See Table E-2 |
| Labdanoic acid unknown #2 | 2283 | $C_{23}H_{38}O_2Si$ | 374 | I | See Table E-2 |
| Labdanoic acid unknown #3 | 2317 | $C_{23}H_{38}O_2Si$ | 374 | I | See Table E-2 |
| Labda-8(20),13-dien-15-oic acid | 2406 | $C_{23}H_{40}O_2Si$ | 376 | P | Cox et al., 2007 |
| **Tetracyclics** | | | | | |
| Beyerene | 1950 | $C_{20}H_{32}$ | 272 | P, L | Adams et al., 2001 |
| Isophyllocladene | 1985 | $C_{20}H_{32}$ | 272 | L | |
| Trachylobane | 2005 | $C_{20}H_{32}$ | 272 | L | |
| Atis-15-ene | 2008 | $C_{20}H_{32}$ | 272 | L | |
| Kaur-15-ene (Isokaurene) | 2012 | $C_{20}H_{32}$ | 272 | L | |
| Phyllocladene | 2032 | $C_{20}H_{32}$ | 272 | L | |
| Atis-16-ene | 2058 | $C_{20}H_{32}$ | 272 | L | |
| Kaur-16-ene | 2062 | $C_{20}H_{32}$ | 272 | L | |
| **Totarols and Sempervirol** | | | | | |
| Sempervirol | 2215 | $C_{23}H_{38}OSi$ | 358 | P | Cox et al., 2007 |
| Totarol | 2323 | $C_{23}H_{38}OSi$ | 358 | P | Cox et al., 2007 |
| 7-Hydroxytotarol | 2443 | $C_{26}H_{46}O_2Si_2$ | 446 | P | Cox et al., 2007 |
| **Other** | | | | | |
| Laurenene | 1891 | $C_{20}H_{32}$ | 272 | P | Adams et al., 2001; Philp, 1985 |
| Cembrene | 1931 | $C_{20}H_{32}$ | 272 | L | |
| Cembrene, <(3E)-> | 1963 | $C_{20}H_{32}$ | 272 | P | Adams et al., 2001 |
| Diterpene unknown #1 | 2021 | $C_{20}H_{32}$ | 272 | I | See Table S2 |
| Diterpenoic acid unknown #1 | 2233 | $C_{23}H_{38}O_2Si$ | 374 | I | See Table S2 |
| Diterpenoic acid unknown #2 | 2253 | $C_{23}H_{38}O_2Si$ | 374 | I | See Table S2 |
| Diterpenol unknown #1 | 2436 | $C_{23}H_{38}O_2Si$ | 372 | I | See Table S2 |

[a] Composition and molecular weights (MW) reported as their trimethylsilyl derivatives.
[b] Method of compound identification: I, interpretation of MS fragmentation patterns; L, Wiley and NIST libraries; S, Standard; P, spectra data published in the literature.

common in Podocarpaceae and Cupressaceae, but rare in other families.

Overall, the distribution of specific compounds and compound structure classes are consistent with a large and exhaustive survey completed by Otto and Wilde (2001). For example, of the molecules often reported to have high taxonomic specificity, we found that phyllocladene was indeed specific to many species in Podocarpaceae, and a few species in Cupressaceae, although concentrations were far higher in Podocarpaceae than Cupressaceae. We also found that beyerenes and kaurenes were widespread with the exception of the Taxaceae. However, in contrast to Otto and Wilde (2001), we detected atisene in one species of Podocarpaceae.

Despite the differences in diterpenoid concentrations between species, even within the same family, it was suggested that terpenoid concentrations are significantly higher for species with evergreen leaf lifespans (Diefendorf et al., 2012). In that study,

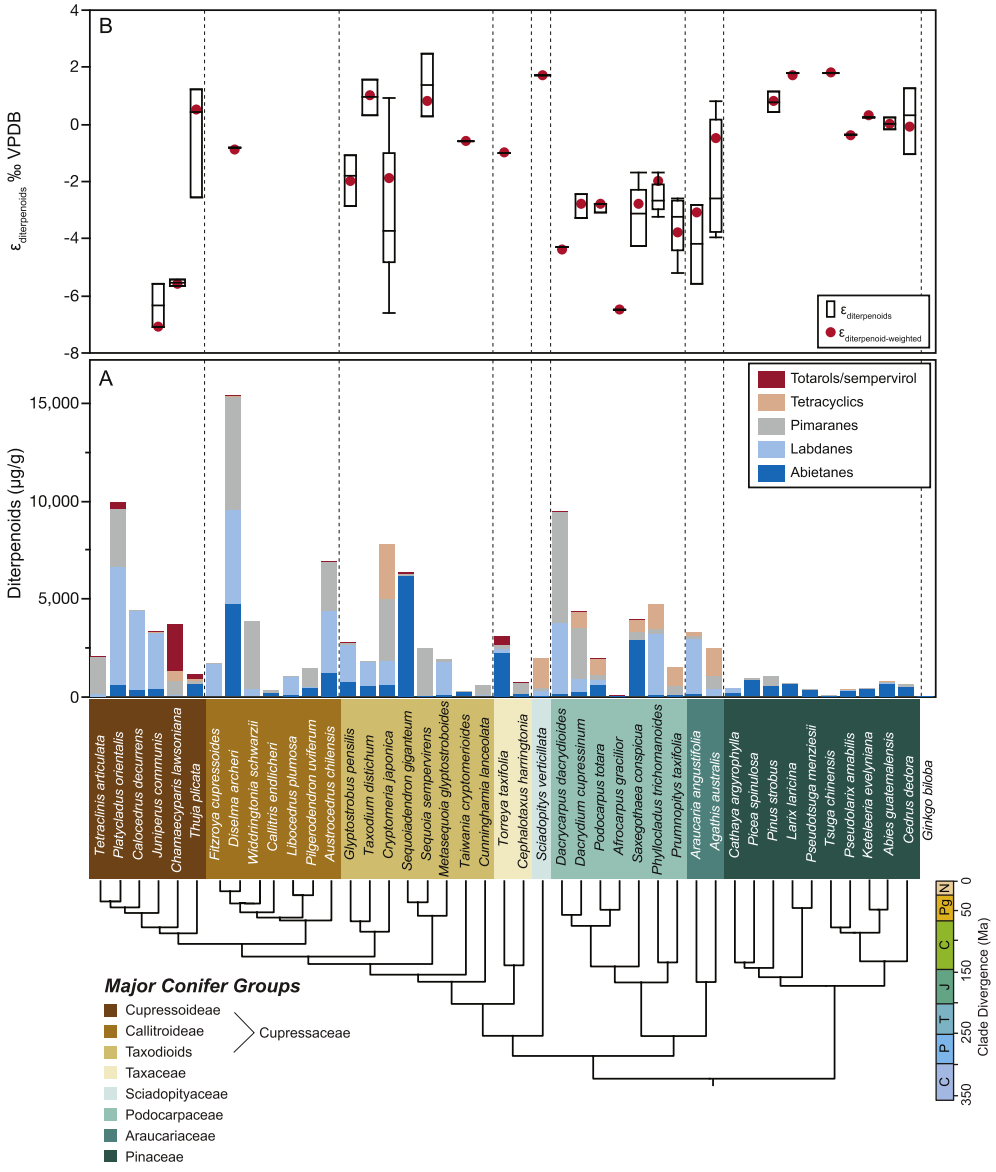


**Fig. 2.** Diterpenoid concentrations in µg/g dry leaf (A) and $\varepsilon_{diterpenoid}$ values (B) for the 43 conifer species and *Ginkgo biloba*. Conifer species are grouped into major clades with a DNA sequenced-based phylogeny that is age-calibrated using the fossil record (Rai et al., 2008; Leslie et al., 2012). The tetracyclic compounds include beyerane, kaurane, phyllocladane. $\varepsilon_{diterpenoid}$ values for individual compounds are represented using box and whisker plots with the median, upper and lower quartiles, and maximum and minimum values indicated. The concentration-weighted $\varepsilon_{diterpenoid}$ values are indicated with red dots. A strong agreement exists between mean $\varepsilon_{diterpenoid}$ values and $\varepsilon_{diterpenoid\text{-}weighted}$ values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 2**
Mean diterpenoid compound concentrations (µg/g) by compound structure class and diterpenoid biosynthetic carbon isotope fraction ($\varepsilon_{diterpenoid\text{-}weighted}$).

| Major conifer group | Diterpenoids µg/g | | | Abietanes µg/g | | | Labdanes µg/g | | | Pimaranes µg/g | | | Tetracyclics µg/g | | | Totarols and Sempervirol µg/g | | | $\varepsilon_{diterpenoid\text{-}weighted}$ (‰, VPDB) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | µ | 1σ | n | µ | 1σ | n | µ | 1σ | n | µ | 1σ | n | µ | 1σ | n | µ | 1σ | n | µ | 1σ | n |
| Araucariaceae | 2910 | 608 | 2 | 66 | 50 | 2 | 1614 | 1752 | 2 | 395 | 346 | 2 | 786 | 857 | 2 | 0 | 0 | 2 | −3.1 | 1.6 | 2 |
| Callitroideae | 5123 | 5583 | 7 | 948 | 1723 | 7 | 1563 | 1816 | 7 | 1842 | 2204 | 7 | 1 | 4 | 7 | 5 | 10 | 7 | −0.9 | | 1 |
| Cupressoideae | 4138 | 3219 | 6 | 331 | 268 | 6 | 2177 | 2552 | 6 | 969 | 1213 | 6 | 91 | 195 | 6 | 499 | 942 | 6 | −4.1 | 3.3 | 3 |
| Pinaceae | 560 | 301 | 10 | 441 | 245 | 10 | 26 | 67 | 10 | 92 | 149 | 10 | 0 | 0 | 10 | 0 | 0 | 10 | 0.6 | 0.8 | 7 |
| Podocarpaceae | 3755 | 3018 | 7 | 564 | 1046 | 7 | 1111 | 1565 | 7 | 1376 | 2095 | 7 | 642 | 475 | 7 | 9 | 11 | 7 | −3.7 | 1.4 | 7 |
| Sciadopityaceae | 2002 | | 1 | 14 | | 1 | 272 | | 1 | 170 | | 1 | 1512 | | 1 | 0 | | 1 | 1.7 | | 1 |
| Taxaceae | 1926 | 1719 | 2 | 1167 | 1492 | 2 | 110 | 151 | 2 | 399 | 257 | 2 | 0 | 0 | 2 | 218 | 302 | 2 | −1.0 | | 1 |
| Taxodiaceae | 3004 | 2733 | 8 | 1049 | 2084 | 8 | 762 | 830 | 8 | 812 | 1257 | 8 | 347 | 982 | 8 | 11 | 29 | 8 | −0.7 | 1.9 | 5 |
| All | 2983 | 3295 | 43 | 648 | 1220 | 43 | 973 | 1531 | 43 | 873 | 1466 | 43 | 254 | 571 | 43 | 84 | 372 | 43 | −1.6 | 2.5 | 27 |

the total number of conifer species was small (limited to species growing in Pennsylvania) and in the Cupressaceae it was limited to only a few deciduous species. In this study, we find no difference in concentrations between deciduous and evergreen species in taxodioid Cupressaceae ($t$-test, $p = 0.4$) and in Pinaceae ($t$-test, $p = 0.8$). This is surprising given the differences in growth strategies and the greater chemical investment in longer-lived leaves (e.g., Coley et al., 1985). These findings are similar to our analysis of the $n$-alkyl lipids in these same samples where we also did not detect any influence of lifespan on concentration (Diefendorf et al., 2015b). Future studies will need to evaluate if leaf lifespan has any influence on diterpenoid investment.

### 3.2. Phylogenetic analysis of diterpenoids

Despite the high degree of variability in diterpenoid concentrations among species, conifers often show phylogenetic structuring at the family level. For example, the Pinaceae has lower total diterpenoid concentrations than all other families and clades (Fig. 2, Table 2). When the analysis is restricted to a comparison between Cupressaceae and Pinaceae, the Cupressaceae have significantly higher concentrations of diterpenoids ($t$-test, $p < 0.01$) than the Pinaceae.

When diterpenoid concentrations are fitted to a model (Brownian motion) of trait evolution given the conifer phylogeny, we find that the distribution of total terpenoid concentrations across the tips of the tree does not fit the model. Thus, total terpenoid concentrations do not appear to contain any phylogenetic signal at this finer scale (Table 3). This also appears to be the case in an analysis of the sum of each of the compound structure classes. The tetracyclic structure class might be an exception where Blomberg's K is marginally significant ($p < 0.04$), but Pagel's $\lambda$ is not significant, suggesting that this class shows an equivocal phylogenetic signal. The PCA analysis of total diterpenoid compound structure concentrations also showed no significant signal in the PC Axis 1 scores.

Several compound classes showed a significant phylogenetic signal, however, when their proportions were analyzed instead of their total concentrations. We found that the proportion of abietanes within each species had significant phylogenetic signal (Table 3), which may be attributed to the high proportions of abietanes in the Pinaceae, the taxodioids, and Taxaceae, and lower proportions in Cupressoideae and Araucariaceae (Fig. 3). We also found significant phylogenetic signal in the proportion of tetracyclic compounds (Table 3), likely due to the high proportions of tetracyclic compounds in the Podocarpaceae and Araucariaceae (which together form a single clade) compared to the other major taxonomic groups (Fig. 3). Explicit tests of phylogenetic signal therefore support the idea that shared evolutionary history can influence two of the common diterpenoid compound structure classes, at least as a proportion of the total diterpenoid compounds produced.

### 3.3. Diterpenoid carbon isotopes and phylogenetic analysis

Of the 44 species studied here, we were able to measure the $\delta^{13}C$ values of aliphatic diterpenoids in 27 species (Fig. 2, Table 2 and Supplementary Table S1). The remaining species were problematic to analyze due to co-elution with other compounds that could not be separated. The number of diterpene compounds measured for each species ranged from 1 to 8 (median = 2). The standard deviation of the terpenes within each species was in the range 0.1–2.5‰, with an average of 1.0‰. We also weighted the $\varepsilon_{diterpenoid}$ values by the concentration and respective $\delta^{13}C$ value of each diterpene measured for a given species. This weighting may not be necessary as it had little effect on $\varepsilon_{diterpenoid}$ values. Species mean $\varepsilon_{diterpenoid}$ values were highly correlated with species $\varepsilon_{diterpenoid\text{-}weighted}$ values (least squares linear regression, $R^2 = 0.96$, $p < 0.0001$).

Three species (*Afrocarpus gracilior*, *Chamaecyparis lawsoniana* and *Juniperus communis*) had very negative $\varepsilon_{diterpenoid\text{-}weighted}$ values compared to other species within those subfamilies (Supplementary Table S1). *Cryptomeria japonica* had exceptionally high variability in $\varepsilon_{diterpenoid}$ values between compounds with values ranging from −6.6 to 0.9‰. *C. japonica* has been measured in two other studies (Chikaraishi et al., 2004; Diefendorf et al., 2012) with a smaller range in values (−5.9 to −3.6‰). Overall, it is unclear what might cause such high variability in *C. japonica*.

On average, $\varepsilon_{diterpenoid\text{-}weighted}$ values were different between major taxonomic groups (Fig. 2, Table 2) with average values ranging from −4.1 to +1.7‰. These values are similar to a previous report that indicated less fractionation in diterpenoids than those reported for long-chain $n$-alkanes for Cupressaceae and Pinaceae (Diefendorf et al., 2012). Of the major taxonomic groups, the Cupressoideae have the lowest $\varepsilon_{diterpenoid\text{-}weighted}$ values (−4.1‰), but with high variability (3.3‰, 1σ). In comparison, the Pinaceae have high values (0.6‰) and low variability (0.8‰, 1σ). In contrast to the diterpenoid concentrations, we found significant phylogenetic signal in the

**Table 3**
An analysis of the phylogenetic signal in diterpenoid compounds, as the sum of compounds and as the proportion of compounds, and in the diterpenoid biosynthetic carbon isotope fraction ($\varepsilon_{diterpenoid}$). Values in bold have $p$-values that are significant (indicated with an asterisk).

| Diterpenoid analysis | Blomberg's K | | Pagel's lambda | |
|---|---|---|---|---|
| | Value | P | Value | P |
| **Sum of diterpenoids** | | | | |
| Phylo PC1 | 0.23 | 0.932 | 0.09 | 0.404 |
| All diterpenoid | 0.17 | 0.964 | 0.11 | 0.224 |
| Abietanes | 0.15 | 0.965 | 0.00 | 1.000 |
| Labdanes | 0.26 | 0.764 | 0.11 | 0.345 |
| Pimaranes | 0.20 | 0.938 | 0.04 | 0.728 |
| Tetracyclics | **0.57** | **0.044*** | 0.54 | 0.159 |
| Totarols and sempervirol | 0.37 | 0.386 | 0.00 | 1.000 |
| **Proportion of diterpenoids** | | | | |
| Phylo PC1 | 0.18 | 0.961 | 0.11 | 0.232 |
| Abietanes | **0.56** | **0.002*** | **0.60** | **0.000*** |
| Labdanes | 0.24 | 0.87 | 0.08 | 0.444 |
| Pimaranes | 0.22 | 0.955 | 0.00 | 1.000 |
| Tetracyclics | **0.98** | **0.001*** | **1.05** | **0.000*** |
| Totarols and sempervirol | 0.40 | 0.359 | 0.00 | 1.000 |
| **$\varepsilon_{diterpenoid}$ values** | | | | |
| $\varepsilon_{diterpenoid\text{-}weighted}$ | **0.68** | **0.018*** | **0.48** | **0.038*** |

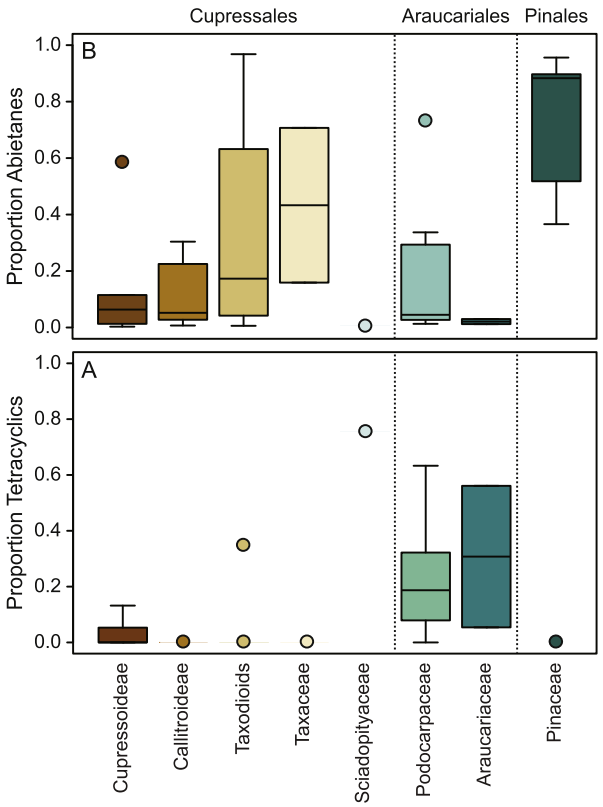


**Fig. 3.** The proportion of diterpenoids as tetracyclic (A) and abietane (B) compound structures in the major conifer groups. Values for species in each of the conifer groups are represented using box and whisker plots with the median, upper and lower quartiles, and maximum and minimum values indicated. Circles indicate outliers or species values when $n \leq 2$. The proportion of the tetracyclic and abietane compound structures contain a significant phylogenetic signal (see Table 3).

$\varepsilon_{diterpenoid\text{-}weighted}$ values (Table 3). We speculate that these differences in $\varepsilon_{diterpenoid}$ values may be related to differences in carbon storage and metabolism between species and families, possibly associated with varying amounts of stored carbon resources being utilized to synthesize isoprene and/or incorporation of isopentenyl diphosphate (IPP) from the MVA pathway (Kreuzwieser et al., 2002; Affek and Yakir, 2003; Diefendorf et al., 2012).

The $\delta^{13}C$ value of diterpenoids can serve as an indicator of conifer $\delta^{13}C$ values (Schoell et al., 1994; Simoneit et al., 1995; Diefendorf et al., 2012, 2015a; Holman and Grice, 2018), which can be useful for comparing the response of conifers and angiosperms, using angiosperm-specific biomarkers, to major climatic events (e.g., Schouten et al., 2007). However, given the large differences in $\varepsilon_{diterpenoid\text{-}weighted}$ between conifer families, it is important to be careful when measuring diterpenoid $\delta^{13}C$ values if taxa are likely to be derived from different conifer groups. For example, if both Podocarpaceae and Pinaceae are present, then variations in the abundance of species or individuals from these two groups could influence sedimentary diterpenoid $\delta^{13}C$ values given their large differences in $\varepsilon_{diterpenoid\text{-}weighted}$ (Table 2). By measuring the most specific diterpenoid compound structures of each clade, as well as incorporating information from the pollen or macrofossil record into geochemical interpretations, it should be possible to account for these phylogenetic differences in $\varepsilon_{diterpenoid}$ values. In the example above, if Podocarpaceae and Pinaceae are both known to be present at a site through pollen records, then it should be possible to measure the $\delta^{13}C$ values of the Podocarpaceae by limiting the analysis to the tetracyclic compounds, as they would be specific to this group.

### 3.4. Diterpenoids as paleovegetation and chemotaxonomic indicators

The high variability in diterpenoid concentrations between different species suggests that paleovegetation reconstruction, based on ratios of conifer diterpenoids and angiosperm-specific biomarkers, would be problematic and highly uncertain. Yet, recent and geologic applications (Bechtel et al., 2005; Diefendorf et al., 2014; Giri et al., 2015; Denis et al., 2017) compare well with independent vegetation estimates (vegetation surveys, pollen, macrofossils). We speculate that mixing from many individuals and species combined with time averaging allows these comparisons to work, at least in a semiquantitative way. For paleovegetation analysis of sediments, we recommend that diterpenoid concentrations still be used (e.g., Diefendorf et al., 2014). When analyzing diterpenoids in sediments, diterpenoid proportions will not be very useful in general because they will be an aggregated value reflecting the conifer species present at a site, the diterpenoid composition and concentration of each of the species present, and taphonomic and diagenetic controls on which diterpenoid compounds are preserved (Bechtel et al., 2003; Nakamura et al., 2010; Diefendorf et al., 2014; Giri et al., 2015). Nevertheless, our results suggest that bulk sediment diterpenoids could prove useful for paleovegetation reconstruction in certain cases; for example, sediments containing abietane compound structures would suggest that Pinaceae was the dominant conifer clade at a site.

The phylogenetic structure in conifer diterpenoids that we identify here, at least in terms of the relative proportions of some compound classes, may be useful for chemotaxonomy. On the other hand, individual taxa within major clades can still show wide variation in their diterpenoid concentrations and the specific compounds present. For example, the closely related species *Sequoiadendron giganteum*, *Sequoia sempervirens*, and *Metasequoia glyptostroboides* in the Cupressaceae subfamily Sequoioideae have dramatically different diterpenoid compositions in their needles. Although fossil and living representatives of particular species typically show similar chemical profiles and thus can be easily compared (Otto et al., 2002, 2003; Simoneit et al., 2016), the variation that exists within clades makes it difficult to unequivocally use chemotaxonomy to identify and place fossils that lack living representatives or whose phylogenetic relationships are uncertain.

When biomarkers are applied to the taxonomic assignment of fossils, particularly those whose family-level relationships are uncertain or those without direct modern representatives, it would be useful to combine several traits such as diterpenoid concentration and proportion, *n*-alkane concentration and average chain length, and measurements of $\varepsilon_{diterpenoid}$. This would require measurements of $\delta^{13}C_{leaf}$ and could be approximated by measuring cuticle $\delta^{13}C$, if preserved (Royer and Hren, 2017). Using such a framework, it should be possible to broadly distinguish major clades such as Cupressaceae, Podocarpaceae, and Pinaceae, although there is enough variability among taxa within these clades that distinctions among them are not foolproof.

Based on our results, it appears that diterpenoid concentrations are very labile and can evolve at a high rate, thus obscuring phylogenetic patterns. Our data would also suggest that relative compound proportions and *n*-alkanes (chain-length, concentration; Diefendorf et al., 2015) change more slowly than the concentrations of diterpenoids and their exact chemical profiles. This would be consistent with conservation in the enzymes required to synthesize the major diterpenoid compound classes, but high rates of modification in the concentration of diterpenoids and high diversity in compound modifications within the same structure class (Langenheim, 1994; Keeling and Bohlmann, 2006; Keeling et al., 2008). This suggests that the proportions of *n*-alkanes and diterpenoids might prove more useful for chemotaxonomy, especially for fossils from earlier in the evolutionary history of conifers.

This kind of phylogenetic approach, but with many more species, may be a way forward to quantify how labile these chemical biomarker traits are. Future research could assess rates of evolution in biomarkers by measuring diterpenoid profiles for all conifer species, then analyzing them in the context of a full species-level phylogeny for conifers (Rai et al., 2008; Leslie et al., 2012).

## 4. Conclusions

The diterpenoid concentrations and compositions from 43 species of conifers, and *Gingko biloba*, growing at a single site in California, were highly variable among species and families. Overall, Pinaceae had lower diterpenoid concentrations than all other families and clades. When diterpenoid concentrations were fitted to a model of trait evolution (Brownian motion), we found that total diterpenoid concentrations lacked a clear phylogenetic signal. However, when we analyzed the results by the proportion of compound class structures within each species, we found significant phylogenetic signal in the abietane and tetracyclic compounds.

Diterpenoid biosynthetic carbon isotope fractionation exhibited a large range in values, although not that dissimilar to those observed for *n*-alkanes in the same samples. Despite the large range in values, we found a clear phylogenetic structure and signal in the $\varepsilon_{diterpenoid}$ values. When using diterpenoids for paleovegetation reconstruction or as conifer $\delta^{13}C$ recorders, it will be important to know what species are present using independent methods, such as with pollen and/or macrofossils, to improve diterpenoid-based paleovegetation reconstructions, conifer $\delta^{13}C$ reconstructions, or chemotaxonomic assignments.

It should be possible to assign an unknown species to the level of Cupressaceae, Podocarpaceae, or Pinaceae. However, we add the caveat that there are enough anomalous taxa in each of these groups that any assignment should be taken with some degree of caution. For chemotaxonomic assignment, we recommend using as many traits as possible given that the phylogenetic signal was limited to the proportion of abietanes, tetracyclics, and $\varepsilon_{diterpenoid}$ values. Based on our results, we recommend against assuming that closely related species would have similar diterpenoid compositions.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at http://dx.doi.org/10.17632/bc5pf223v5.1.

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