Defining phytochemical phenotypes: size and shape analysis of phenolic compounds in oaks (Fagaceae, *Quercus*) of the Chihuahuan Desert

Christopher T. Yarnes, William J. Boecklen, Kati Tuominen, and Juha-Pekka Salminen

Abstract: Interspecific variation in phenolic metabolism across plant species has been correlated to numerous ecological factors, yet generalities concerning the ecological role(s) of phenolics remain elusive. Moreover, studies of allometric variation (absolute and relative concentration) in phenolic metabolism are rare despite the importance of each to ecological interactions. In this study, we characterize individual phenolics in a group of 12 oak species from the Chihuahuan Desert and examine interspecific variation in the absolute and relative concentrations of phenolics using size and shape analysis. Size and shape analysis was able to successfully identify those compounds that contribute most to the interspecific allometric variation in phenolics and classify the oak species on the basis of phenolic metabolism. White versus black oak subgenera were found to be most different in their phenolic composition, where the two black oak species contained fewer and less diverse phenolics. Within the 10 white oak species, a predominantly eastern white oak, *Quercus muhlenbergii* Engelmann, was found to vary significantly from the more widespread white oaks of the Chihuahuan Desert. We also report the occurrence of complex tannins in oaks of North America for the first time, update proposed pathways of ellagitannin biosynthesis in oaks, and discuss the applicability of size and shape analysis to ecological studies of phenolics.

Key words: phenolic, size and shape, relative, absolute, *Quercus*, oak, phenotype.

Résumé : La variation interspécifique du métabolisme phénolique, parmi les espèces de plantes, a été corrélée avec de nombreux facteurs écologiques, alors que les généralités concernant le(s) rôle(s) écologique(s) des phénols demeure(nt) obscur(es). De plus, les études sur la variation allométrique (concentration absolue ou relative) du métabolisme phénolique sont rares, en dépit de l'importance de chacune des interactions écologique. Les auteurs caractérisent les substances phénoliques chez un groupe de 12 espèces de chênes du Désert de Chihuahuan et examinent la variation interspécifique dans les concentrations absolues et relatives des phénols, en utilisant l'analyse des dimensions et des formes. L'analyse des dimensions et des formes permet d'identifier avec succès les composés qui contribuent le plus à la variation allométrique interspécifique et de classifier les espèces de chênes sur la base du métabolisme phénolique. On constate que les sous genres, noirs vs blancs, diffèrent le plus dans leurs compositions phénoliques, et que les deux espèces de chênes noirs contiennent des phénols moins abondants et moins diversifiés. Parmi les 10 chênes blancs, on constate qu'un chêne blanc plus abondant dans l'est, le *Quercus muhlenbergii* Engelmann, diffère significativement des chênes blancs plus répandus du Désert de Chihuahuan. Les auteurs mentionnent également, pour la première fois, la présence de tannins complexes dans les chênes de l'Amérique du Nord, font une mise à jour des sentiers proposés pour la biosynthèse de l'ellagitannin chez les chênes, et discutent l'applicabilité de l'analyse des formes et des dimensions dans les études écologiques sur les phénols.

Mots clés : phénols, dimension et forme, relatif, absolu, *Quercus*, chêne, phénotype.

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Introduction

Phenolics represent a diverse group of plant compounds, widely distributed throughout the plant kingdom (Haslam 1989; Buckingham 1993). Variation in phenolics has been correlated with an equally diverse set of ecological factors,

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such as UV radiation (Lavola 1998), excess photochemical energy (Grace and Logan 2000; Close and McArthur 2002), allelopathy (Putnam and Tang 1986), resource availability (Bryant et al. 1983; Herms and Mattson 1992), microbes and pathogens (Dudt and Shure 1993; Latte and Kolodzeij 2000), herbivores (e.g., Feeny 1970; Nyman and Julkunen-Tiitto 2000; Abrahamson et al. 2003), and recently with larger scale processes such as community dynamics (Bailey et al. 2004) and ecosystem function (Kraus et al. 2003; Schweitzer et al. 2004). Yet many empirical studies of phenolics have relied on summary or indirect measures of phenolics, or a limited subset of the phenolics found within the plant (see Waterman and Mole 1994 for techniques). While simple and inexpensive, these measures fail to capture the diverse structure-dependent functionality of individual phenolics (Zucker 1983; Ayres et al. 1997) or their activity as complex mixtures (Ayres et al. 1997; Barbehenn et al. 2005). Even more troublesome, measurements of total phenolics may not represent the sum of the concentration of individual phenolics (Appel et al. 2001; Salminen 2003; Salminen et al. 2004). The measurement of individual phenolic compounds (Ossipov et al. 1995, 1996; Salminen et al. 1999; Karonen et al. 2004) is required to appropriately characterize variation in the phenolic phenotypes of plants.

Individual phenolics exhibit complex patterns of bioactivities and ecological function, apparently due to compound-specific differences in phenolic structure (Zucker 1983). Structural differences may determine rates of radical scavenging (Yamasaki et al. 1997; Hagerman et al. 1998), protein precipitation (Scalbert 1991) and metal chelation (Mila et al. 1996), as well as levels of toxicity (Nishizawa et al. 1990; Ayres et al. 1997) and rates of prooxidant activity in consumers (Barbehenn et al. 2005). Structural aspects of phenolic activity may also explain a portion of the variation in ecological factors to which summary phenolics have been correlated (Kraus et al. 2003). Relative concentrations of plant secondary compounds may also be very important to determining defense capabilities of particular phenotypes (Rasmussen and Einhellig 1977; Espinosa-Garcia and Langenheim 1991; Stamp and Yang 1996; Castellanos and Espinosa-Garcia 1997; An et al. 2001; Barbehenn et al. 2001) and variation in plant chemical defenses (McKey 1979; Berenbaum 1988; Langenheim 1994) may influence the multidimensional patterns of abiotic and biotic interactions among plants (Linhart 1991). It has become increasingly evident that, to obtain generalities surrounding the role(s) of phenolics in plant ecology, consideration must be given to the complex variation in absolute concentration (AC) and relative concentration (RC) of phenolics in plants.

Relating variation in the absolute and relative production of individual phenolics within plants, or phenolic phenotypes, to genetic and ecological components poses a considerable analytical problem. Typically, standard ordination techniques, such as principal components analysis (PCA) and cluster analysis (parametric and nonparametric), are used to describe variation in metabolite production (e.g., Chen et al. 2003; Brenes-Arguedas and Coley 2005) and relate this variation to ecological factors (e.g., Keinanen et al. 1999; Zangerl and Berenbaum 2003). However, these techniques do not partition the total variation in the concentrations of metabolites into that attributed to variation in the AC, RC, and the co-variation of the AC and RC of metabolites in plants.

Size and shape analysis provides the conceptual framework and analytical tools to examine variation and co-variation in the AC and RC of phenolics in plants (see Mosimann and James 1979; Boecklen and Price 1989), while also allowing appropriate methods to relate this variation to co-variation in ecological factors (Darroch and Mosimann 1985; Boecklen et al. 1991). Mosimann (1970) defines size and shape variables that are geometrically meaningful, have well-known sampling distributions, and are suitable for separate or joint analysis by standard multivariate techniques. Further, total variation in size and shape can be partitioned into variance components that represent variation in shape and variation in size. For example, the sum of the eigenvalues from discriminant analysis on the size and shape variables gives the percent variation of size and shape that is due to size alone (Darroch and Mosimann 1985). Previously, size and shape analysis has been used to relate variation in the AC and RC of minerals in plants to co-variation in sawfly densities (Boecklen et al. 1991). Size and shape analysis provides a more complete definition of the phytochemical (phenolic) phenotype and provides a framework for examining its co-variation with ecological factors and processes.

The oaks of Mexico and the southwestern United States are exposed to abundant heterogeneity in both abiotic and biotic forces (Boecklen and Spellenberg 1990; Aguilar and Boecklen 1992; Gaylord et al. 1996; Preszler et al. 1996) that have been shown to significantly affect phytochemistry (Bihr 2000; Yarnes and Boecklen 2005, 2006). In this study, size and shape analysis was used to quantify variation within ACs and RCs of individual phenolics in 10 species of white oaks (*Quercus* sect. *Quercus*) and two species of black oaks (*Quercus* sect. *Lobatae*) common to New Mexico and Arizona. Individual phenolics in these 12 oaks including phenolic acids, hydrolysable tannins, and flavonoid glycosides were quantified using a current analytical tool: high-performance liquid chromatography (HPLC) diode-array detection (DAD) electrospray-ionization (ESI) mass spectrometry (MS) (HPLC-DAD-ESI-MS). The utility of size and shape analysis in constructing phytochemical phenotypes and relating variation in phytochemistry to variation in the ecology of oaks is discussed.

Materials and methods

The oaks

The so-called white oaks are members of the subgenus *Quercus* sect. *Quercus* (Nixon 1993). The center of biodiversity for this subgenus in North America lies within Mexico, the southwestern United States, and Central America. They may be trees or shrubs, evergreen or deciduous; the bark ranges across many different colors and textures. Basic distinctions of the subgenera *Quercus* from sections *Lobatae* (black oaks) and *Protobalanus* (golden-cup oaks) are often provided by the morphological differences in toothed margins (from sect. *Lobatae*), and duration of fruit maturation (from sect. *Protobalanus).* In section *Quercus*, while leaf margins may be entire or toothed, if teeth are present they

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are never bristle-tipped. Acorns mature annually, not biennially.

Ten species of the most common white oaks of the southwestern United States were selected for this study and two black oaks were selected as outgroups for the comparative analysis of the 10 white oaks (Table 1). Reference specimens for all species except *Q. grisea* and *Q. gambelii* (CY 2004-1 to 2004-10) were deposited in the herbarium at New Mexico State University (NMC). *Quercus grisea* and *Q. gambelii* trees from this study were described by Howard et al. (1997).

Sample collection and preparation

Ten trees of each species were selected at their respective locations during 22–24, 27–28, and 30 June 2004 (Table 1). Ten leaves from each tree were selected from the lower braches; obvious "sun" leaves (hard, waxy leaves) or any second-year leaves were avoided to minimize within-tree variation of samples (Feeny 1970). Leaves were placed on ice in envelopes for transport to the laboratory. Leaves were air-dried on a set of shelves in a ventilated fume hood. Air-drying is not the optimal drying method (Salminen 2003), but is known to alter levels of hydrolysable tannins and flavonoid glycosides only slightly in *Q. robur* (Salminen et al. 2004). Air-dried oak leaves were then ground to a fine powder using a ball mill (Wig-L-Bug, Reflex Analytical, New Jersey, USA) and pooled within trees. After grinding, phenolics were extracted three times from leaf tissue (approx. 20 mg dry mass) with 70% aqueous acetone + 0.1% ascorbic acid (added to prevent oxidation of phenolics). This extraction solvent was found to provide better recovery over a wide range of aqueous solvents (methanol: 50% MeOH, 70% MeOH, 85% MeOH; acetone: 50% Me_2CO, 70% Me_2CO, 85% Me_2CO; Tuominen et al. 2005). Extracts were loosely covered with aluminum foil and placed in a closed fume hood overnight to remove the acetone fraction. The air-dried extracts were then dissolved in 1 mL water, the supernatants centrifuged (10 min at 5000*g*), filtered through a 0.45 μm PTFE (polytetraflouroethylene) filter, and kept frozen at –20 °C until analysis with HPLC-DAD-ESI-MS.

Isolation and identification of castavaloninic and vescavaloninic acids

The structural characteristics and identities of two acyclic isomeric ellagitannins, castavaloninic acid and vescavaloninic acid (molecular weight 1102), were elucidated through nuclear magnetic resonance (NMR) analysis following fractionation and purification from crude extracts. The isomeric compounds were isolated using a combination of Sephadex LH-20 (450 mm × 25 mm) and Merck LiChroPrep RP-18 (600 mm × 25 mm, 40–63 μm) columns following methods outlined by Salminen et al. (1999, 2001). The NMR spectra of castavaloninic acid and vescavaloninic acid were acquired using a Bruker Avance 500 spectrometer (equipped with broadband observe (BBO)-5 mm-Zgrad probe) operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C. Spectra were recorded at 25 °C using acetone-d_6 + D_2O as a solvent and tetramethlysilane (TMS) as an internal reference (0.00 ppm). In addition to basic proton and carbon spectra, 2-D techniques including double-quantum filtered correlation spectroscopy (DQF-COSY), nuclear Overhauser effect Spectroscopy (NOESY), heteronuclear multiple-quantum correlation (HMQC), and heteronuclear multiple-bond correlation (HMBC) were used. These spectra were constructed through standard gradient-enhanced pulse programs installed by Bruker.

Analysis of hydrolysable tannins and other phenolic compounds

Individual hydrolysable tannins (both galloylglucoses and ellagitannins), flavonoid glycosides, and simple phenolics were measured following the methods outlined in Salminen et al. (1999). The water-soluble phenolic compounds of oak leaves were analyzed under negative electrospray ionization with a HPLC-DAD-ESI-MS system (Waters 2795 separations module with 2996 DAD, Milford, Massachusetts, USA; Micromass ZMD 2000, Manchester, UK) at 280, 315, and 349 nm. Two solvents were used: (A) 0.1% formic acid (HCOOH) in H_2O and (B) 0.1% HCOOH in acetonitrile (MeCN). The elution profile was: 0–3 min, 100% A (isocratic); 3–30 min, 0%–30% B in A (linear gradient); 30–40 min 30%–45% B in A (linear gradient). Flow rate used was 1 mL·min^{-1}. The HPLC column used was a Merck Superspher 100 RP-18 column (75 mm × 4.0 mm i.d., 4 μm, Darmstadt, Germany). The following conditions were used in negative-ion ESI-MS: capillary voltage, –2.75 kV; cone voltage, –43 V; extractor voltage, –5 V; desolvation temperature, 350 °C; desolvation gas flow rate, 550 L·h^{-1}. The DAD was operated at 280, 315, and 349 nm.

Individual compounds were identified on the basis of UV and mass spectra, and retention times. Simple phenolics, hydrolysable and complex tannins, and flavonoid glycosides were quantified using chlorogenic acid, pentagalloylglucose, and kaempferol as external standards. Pure compound peaks were quantified with DAD; in the case of overlapping compound peaks, they were quantified selectively with ion trace analysis of their deprotonated molecules as described in Salminen et al. (2001). Differences in the ionization between individual runs were standardized using 6-bromo-2-naphthyl-β-D-glucopyranoside as an internal standard.

An attempt was made to quantify proanthocyanidin oligomers (monomers through decamers) and polymers (greater than decamers) from extracts of each species under normal-phase HPLC (NP-HPLC-DAD) as outlined in Karonen et al. (2004). However, because of the chromatographic overlap of proanthocyanidins with the abundant ellagitannins in these oaks, it was impossible to quantify proanthocyanidins through NP-HPLC-DAD. As a result, this group of phenolics was necessarily excluded from our study.

Statistical analysis

We compared the concentrations of 23 phenolic compounds among the 12 species with a one-factor multivariate analysis of variance (MANOVA) with species as the main effect and trees as replicates. We used this design for the 23 $\log_e$+1-transformed concentrations and for the 22 shape variables (see below). We partitioned the MANOVA by phenolic compounds and compared species using a one-factor analysis of variance (ANOVA). We did this largely as a descriptive device to determine which variables contributed most to the MANOVA results. We used *p* values from the

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Table 1. Oak species collection and identification information.

Species	Text references[a]	Common name	Collection date and location
Quercus arizonica Sargent	1/QA	Arizona white oak	June 22; Peloncillo Mtns, New Mexico, USA; 31°31′N, 108°58′W; 1478 m
Quercus gambelii Nuttall	2/QGA	Rocky Mountain white oak	June 24; San Mateo Mtns, New Mexico, USA; 33°55′N, 107°31′W; 2055 m
Quercus grisea Liebmann	3/QGR	Gray oak	June 24; San Mateo Mtns, New Mexico, USA; 33°55′N, 107°31′W; 2055 m
Quercus havardii Rydberg	4/QHA	Havard oak or scrub oak	June 27; Eddy County, New Mexico, USA, along NM 360, 8.0 mi. from US 82
Quercus mohriana Buckley	5/QMO	Mohr oak	June 28; Guadalupe Mtns, New Mexico, USA; Forest Rd. 201/540
Quercus muhlenbergii Engelmann	6/QM	Chinkapin oak	June 28; Guadalupe Mtns, New Mexico, USA; North Fork McKittrick Canyon
Quercus oblongifolia Torrey	7/QO	Mexican blue oak	June 23; Chirichahua Mtns, Arizona, USA; 31°52′N, 109°14′W; 1752 m
Quercus pungens Liebmann	8/QP	Sandpaper oak	June 27; Guadalupe Mtns, New Mexico, USA, NM 137 mile marker 28.
Quercus rugosa Needham	9/QR	Netleaf oak	June 23; Chirichahua Mtns, Arizona, USA; 31°52′N, 109°14′W; 1752 m
Quercus turbinella Greene	10/QT	Scrub live oak	June 30; Florida Mtns, New Mexico, USA; Rockhound State Park Campground
Quercus emoryi Torrey	11/QE	Emory oak	June 23; Chirichahua Mtns, Arizona, USA; 31°52′N, 109°14′W; 1752 m
Quercus hypoleucoides Camus	12/QH	Silverleaf oak	June 23; Chirichahua Mtns, Arizona, USA; 31°52′N, 109°14′W; 1752 m

[a]Text references represent the numbers and abbreviations used within text and tables.

ANOVAs to measure the strength of the contribution and did not consider individual ANOVAs to be independent tests. We repeated all analyses for phenolics in white oaks alone (excluding *Q. muhlenbergii*) and for ellagitannins, the most abundant phenolic in our study, in white oaks alone.

We used size and shape analysis (Mosimann 1970; Mosimann and James 1979; Boecklen and Price 1989; Boecklen et al. 1991) to compare oak species on the basis of the AC and RC of foliar phenolic compounds. First, we compared species with respect to $\log_e$+1-transformed concentrations (size). We calculated the geometric mean of the size variables and then created shape variables by subtracting from each $\log_e$+1-transformed phenolic compound concentration the $\log_e$+1 geometric mean of all 23 concentrations. Because the rank of the matrix of shape variables is one less than that for the matrix of size variables, we excluded the variable $\log_e$(coumaroylquinic acid)-$\log_e$(geometric mean) from the analysis. This compound was chosen for exclusion because it was the most invariant across species and did not contribute heavily to the discriminant functions for size. For the ellagitannin analysis, we excluded $\log_e$(acutissimin A) – $\log_e$(geometric mean) from the analysis as it was the most invariant of the ellagitannins in white oaks.

We then compared the AC and RC of phenolic compounds in oak species with discriminant function analyses (DFA). The DFA indicated whether species differed in the AC and RC of phenolic compounds and also provided information about how oak species differed. We also used the ratio of the Eigenvalues to determine the relative contribution of size (AC) and shape (RC) variables to the classification of species. Analyses were carried out using SYSTAT 10.2 (2000; SPSS, Chicago, Illinois, USA).

Results

Identification of individual compounds

We identified 23 individual phenolic compounds in white and black oaks of the Chihuahuan Desert using negative-ion HPLC-DAD-ESI-MS (Table 2). Compounds were identified on the basis of their retention times (R_t), UV spectra and mass spectral characteristics; for two compounds NMR was also used. Four phenolic acids were identified as galloylquinic acid (**1**), coumaroylquinic acid (**2**), chlorogenic acid isomer (**3**), and chlorogenic acid (**4**) (Ossipov et al. 1996). Eight phenolics were identified as ellagitannins: vescavaloninic acid (**5**), castavaloninic acid (**6**), vescalagin (**7**), mongolinin A (**8**), pedunculagin (**9**), castalagin (**10**), acutissimin A (**11**), and cocciferin D_2 (**12**) (Ishimaru et al. 1988; Nonaka et al. 1990; Konig et al. 1994; Salminen et al. 1999; Quideau et al. 2003; Salminen et al. 2004). The separation between vescavaloninic and castavaloninic acids (the determination of the configuration of C-1 of the sugar moiety) was done with ^{1}H- and ^{13}C-NMR; the signals from sugar atoms were assigned by the aid of DQF-COSY, NOESY, and HMQC (heteronuclear single quantum correlation) spectra. The main differences in ^{1}H and ^{13}C chemical shifts were observed at positions 1, 2, and 3. Also, the proton–proton coupling constant $J_{1,2}$ is much smaller in vescavaloninic acid (5.0 Hz) than in castavaloninic acid (2 Hz). The absolute stereochemistry of these compounds has been studied previously in detail by Nonaka et al. (1990), and the NMR characteristics that we found are in close agreement with those reported by them (Nonaka et al. 1990). Four compounds (**13**, **14**, and **15a, 15b**) remained classified as ellagitannins, since their chemical structures could not be fully determined. Seven flavonoid glycosides were classified as

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Table 2. Compound information for phenolics in oaks of the Chihuahuan Desert.

Category and compound	R_t (min)	M_r (g·mol^{-1})	UV max.	*m/z*	Species
Phenolic acids					
1 Galloylquinic acid	8.1	344	210	343, 687, 169, 191	11–12
2 Coumaroylquinic acid	12.2	338	210	337, 191	1–12
3 Chlorogenic acid isomer	13.0	354	210	353, 191	5, 8
4 Chlorogenic acid	13.2	354	210	353, 707, 191	5–8, 10–12
Ellagitannins					
5 Vescavaloninic acid	6.4	1102	205, 260–280sh.	1101, 1084, 1066	1–10
6 Castavaloninic acid	7.5	1102	205, 260–280sh.	1101	1–10
7 Vescalagin	7.7	934	220, 260–280sh.	933, 915, 457	1–12
8 Mongolinin A	7.9	1374	208, 260–280sh.	1373, 1101, 933, 915, 289	2–4, 6, 8, 10
9 Pedunculagin (anomeric mixture)	8.8, 11.3	784	215, 260–280sh.	783, 391, 301	1–10
10 Castalagin	9.5	934	220, 260–280sh.	933, 466	1–12
11 Acutissimin A/B	12.7	1206	206, 260–280sh.	1205, 933, 915, 289	1–10
12 Cocciferin D_2	14.8	1870	206, 260–280sh.	1869, 934	1–10
13 Ellagitannin	17.6	1084	210, 260–280sh.	1083, 1067, 533, 301	1–4, 5–10
14 Ellagitannin	12.3	1326	208, 260–280sh.	1326, 301	2–4, 6, 8, 10
15a, 15b Ellagitannin	19.8, 20.3	1724	212, 260–280sh.	1723, 861	11–12
Flavonoid glycosides					
16 Quercetin glycoside	19.7	464	219, 253, 360	463, 927, 301	1–10
17 Quercetin glycoside	20.1	464	219, 253, 360	463, 927, 301	1–4, 6–8, 10
18 Kaempferol glycoside	21.8	448	218, 250, 352	447, 895, 285	1–10
19 Kaempferol glycoside	23.1	448	218, 250, 350	447, 895, 285	2, 3, 5
20 Flavonoid glycoside	19.2		218, 265, 348		1–10
21 Flavonoid glycoside	21.4		218, 268, 348		1–10
22 Flavonoid glycoside	22.1		218, 268, 351		1–12

Note: R_t, retention time; M_r, molecular weight; UV max., wavelength of UV maximum and any shoulders (sh.); *m/z*, characteristic fragments in ESI-MS; Species, species occurrences.

quercetin glycosides (**16**, **17**), kaempferol glycosides (**18**, **19**), or simply as flavonoid glycosides (**20, 21, 22**) (Ossipov et al. 1995).

Ellagitannins were found to be the most diverse and abundant set of phenolic compounds in this group of white oaks from the Chihuahuan Desert. As such, we focused on the identification and biosynthesis of hydrolysable tannins. Notably, the other major group of hydrolysable tannins, that is, gallotannins, were not represented within the oaks of this study. Complex hydrolysable tannins **8** and **11** were also documented for the first time in the foliage of oaks native to North America. These tannins, also referred to as flavono-ellagitannins or procyanidino-ellagitannins (Khanbabaee and van Ree 2001), are composed of a flavonoid or catechin unit linked to a hydrolysable tannin moiety.

A preliminary biosynthetic pathway for ellagitannins in oak has been outlined from *Q. robur* (Salminen et al. 2004); we extended the pathway to include products found in Chihuahuan Desert white oaks (Fig. 1). Briefly, acutissimin A (**11**) formation results from the attachment of (+)catechin at C-1 on the acyclic glucose core of vescalagin (hydrolysable tannin moiety) following the loss of water through a condensation reaction (Quideau et al. 2003). Mongolinin A (**8**) results through a similar process, except with vescavaloninic acid moiety in place of vescalagin (Ishimaru et al. 1988). Notably, **8** could also result from **11** through the addition of a galloyl group, thus forming a valoneoyl group in **8**.

Size of foliar phenolic compounds among all oaks

The average concentration of individual phenolics was highly variable both within and across oak species (Table 3). Mean ACs of simple phenolics ranged from 0 to 6.64 mg·g^{-1}, ellagitannins from 0 to 12.95 mg·g^{-1}, complex ellagitannins from 0 to 2.28 mg·g^{-1} (these are also ellagitannins by definition), and flavonoid glycosides from 0 to 6.17 mg·g^{-1}. Mean concentrations of phenolics did not vary in unison across species: variation exists in both AC and RC (Fig. 2). The black oaks (*Quercus* sect. *Lobatae*), QE and QH, produced strikingly different types and amounts of phenolics than did the white oaks (*Quercus* sect. *Quercus*). Black oaks were found to contain detectable levels of only 9 phenolics compared with 21 produced by white oaks (Table 3). Unique to the black oaks were the simple phenolic, galloylquinic acid (**1**), and an isomeric ellagitannin (**15a, 15b**; Table 2). QM, a predominantly eastern white oak, was found to have higher ACs of ellagitannins **7** and **9** and lower ACs of **5** and **20** than did its southwestern counterparts; QM did not produce detectable amounts of **13** or **14** and displayed dissimilar profiles of phenolic acids and flavonoid glycosides as well. Because of the dissimilarity between QM and the other white oaks, QM was excluded from subsequent size and shape analyses along with the black oaks when examining phenolics in southwestern white oaks.

MANOVA indicated that ACs of phenolics significantly varied across all oak species ($p < 0.001$, Table 4). Univariate analyses of all 24 $\log_e$+1 ACs across species were significant (all $p < 0.001$, except compound **2**, which had $p = 0.04$).

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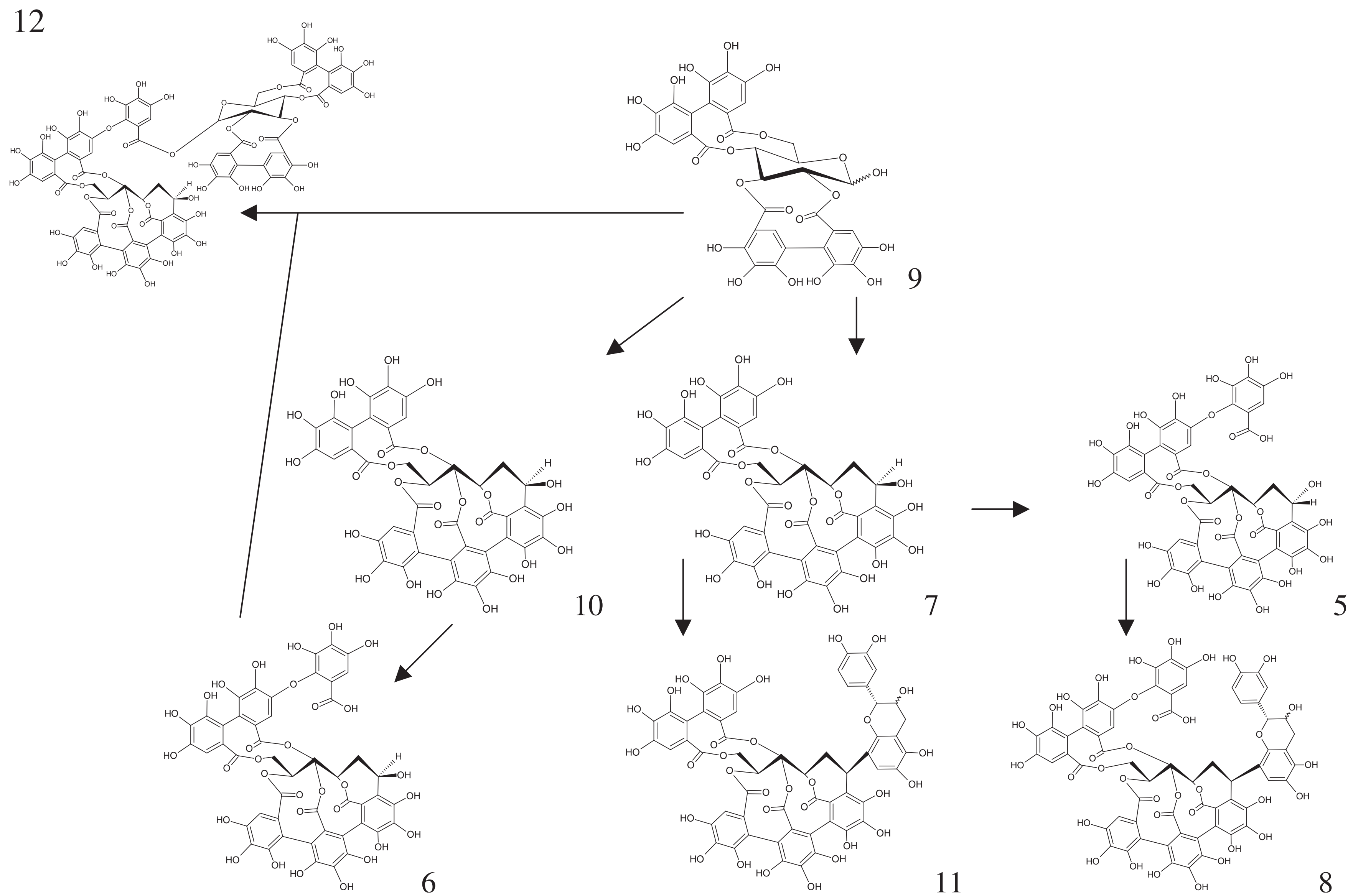
Fig. 1. Proposed biosynthetic relationships among ellagitannins and complex tannins in white oaks of the Chihuhuan Desert. See Table 1 for compound enumeration.



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Variation among all oak species in ACs of phenolics produced 11 significant discriminant functions. The first three discriminant axes accounted for 85% of the variation among species (Table 4, Fig. 3A). The first axis accounted for approximately 54% of the variation and principally represented decreasing concentrations of chlorogenic acid (**4**), galloylquinic acid (**1**), and ellagitannins **15a** and **15b** (Table 5). The first discriminant axis based on the size variables primarily separated black oaks from white oaks (Fig. 3A). The second axis accounted for approximately 20% of the variation and represented decreasing amounts of chlorogenic acid (**4**), chlorogenic acid isomer (**3**), quercetin glycoside (**16**), and vescalagin (**7**) (Table 5). The second axis separated QM from the black oaks and the rest of the white oaks (Fig. 3A). The third discriminant axis explained an additional 11% of the variation and chiefly represented increasing concentrations of ellagitannin (**13**) and kaempferol glycoside (**19**), and decreasing concentrations of mongolinin A (**8**) (Table 5). The third axis served to separate the remaining white oaks (minus QM) of the Chihuahuan Desert. The vast majority of the variation in the AC of phenolics among all species (74%; DF1+DF2) was accounted for by variation between the white oaks and black oaks, and by QM versus all other oaks.

Shape of foliar phenolic compounds among all oaks

MANOVA indicated that RCs of phenolics significantly varied across all oak species ($p < 0.001$, Table 4). Univariate analyses of all 23 $\log_e$+1 RCs were highly significant (all compounds $p \leq 0.001$).

Variation among all oak species in RCs of phenolics produced 11 significant discriminant functions. The first three discriminant axes accounted for 85% of the variation among species (Table 4, Fig. 3B). The first axis accounted for approximately 53% of the variation and represented decreasing RCs of chlorogenic acid (**4**), galloylquinic acid (**1**), and ellagitannins **15a** and **15b** (Table 5). The first discriminant axis of the shape variables separated the black oaks (QH, QE) and QM from the rest of the white oaks (Fig. 3B). The second axis accounted for approximately 21% of the variation and represented increasing RCs of acutissimin A (**11**), quercetin glycoside (**17**), and flavonoid glycoside (**22**), and decreasing amounts of chlorogenic acid isomer (**3**), chlorogenic acid (**4**), and quercetin glycoside (**16**). The second discriminant axis principally separated QM and the black oaks from the rest of the white oaks (Fig. 3B). The third discriminant axis explained an additional 12% of the variation and chiefly represented increasing RCs of ellagitannin **13** and kaempferol glycoside (**19**), and decreasing relative amounts of chlorogenic acid isomer (**3**) and mongolinin A (**8**). The third axis served to separate the white oaks (minus QM) of the Chihuahuan Desert. Similar to the AC of phenolics, the majority of the variation in the RC of phenolics among all species (74%; DF1+DF2) was represented by variation between the white oaks and black oaks, and by *Q. muhlenbergii* versus all other oaks.

The majority of the total variation among oak species in the $\log_e$+1 concentrations of phenolics could be attributed to variation in the $\log_e$+1 RCs. The ratio of the sum of the eigenvalues from discriminant analyses on the absolute and RCs indicates that approximately 183.42/190.87 = 96.1% of the total variation in phenolics among species was due to variation in the RCs of phenolics.

Size of foliar phenolic compounds among white oaks

A MANOVA of the AC of phenolics in white oaks (excluding the black oaks and QM) found ACs of phenolics to vary significantly across species ($p < 0.001$, Table 4). Univariate results indicate that ACs in 19 of the 21 phenolics were significantly different across white oak species (all $p < 0.001$, except $p = 0.08$ for **2**, $p = 0.13$ for **11**, $p = 0.02$ for **20**).

Variation among white oak species (excluding QM) in ACs of phenolics produced eight significant discriminant functions. The first three discriminant axes accounted for approximately 90% of the variation among species (Table 6, Fig. 3C). The first axis accounted for approximately 50% of the variation, accounted for by decreasing concentrations of chlorogenic acid isomer (**3**) and ellagitannin (**14**). The first discriminant axis of the size variables primarily separated QP from the rest of the white oaks (Fig. 3C). The second axis accounted for approximately 30% of the variation and represented decreasing concentrations of chlorogenic acid isomer (**3**), chlorogenic acid (**4**), castavaloninic acid (**6**), mongolinin A (**8**), pedunculagin (**9**), and acutissimin A (**11**) versus increasing concentrations of all other compounds. The second discriminant axis separated QGA and QGR from the rest of the white oaks (Fig. 3C). The third discriminant axis explained an additional 10% of the variation and chiefly represented increasing concentrations of pedunculagin (**9**), castalagin (**10**), ellagitannin (**14**), quercetin glycosides **16** and **17**, flavonoid glycoside (**22**), and decreasing concentrations of flavonoid glycoside (**21**). The third axis served to separate QHA, QMO, and QT from QA and from QR and QO (Fig. 3C).

Shape of foliar phenolic compounds among white oaks

A MANOVA of the RCs of phenolics in white oaks found phenolics to vary significantly across species ($p < 0.001$, Table 4). 19 of the 20 $\log_e$+1 RCs of phenolics were significantly different across the white oaks ($p < 0.001$); only RCs of flavonoid glycoside **20** did not significantly vary between species ($p = 0.222$).

Variation among white oak species in RCs of phenolics produced eight significant discriminant functions. The first three discriminant axes accounted for 88% of the variation among species (Table 4, Fig. 3D). The first axis accounted for approximately 41% of the variation (Table 6) and chiefly represented decreasing RCs of chlorogenic acid isomer (**3**). The second axis accounted for approximately 35% of the variation and was represented by increasing RCs of castavaloninic acid (**6**), mongolinin A (**8**), and pedunculagin (**9**), as well as decreasing relative amounts of ellagitannin **12** and ellagitannin **13**. The third discriminant axis explained an additional 12% of the variation and chiefly represented increasing RCs of castalagin (**10**) and flavonoid glycoside **22** and decreasing RCs of vescalagin (**7**), cocciferin D_2 (**12**), and flavonoid glycoside (**21**). As with the ACs of phenolics in white oaks, the first discriminant axis separated QP, the second discriminant axis distinguished QGA and QGR from the remainder, while the third axis served to generally separate QHA, QMO and QT from QA, and from QR and QO (Fig. 3D).

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Table 3. Least-square mean concentrations (mg·g^{-1} ± SE) of individual phenolic compounds in oak (*Quercus*) species of the Chihuahuan Desert.

Phenolic compound	*Q. arizonica*	*Q. gambelii*	*Q. grisea*	*Q. havardii*	*Q. muhlenbergii*	*Q. mohriana*	*Q. oblongifolia*	*Q. pungens*	*Q. rugosa*	*Q. turbinella*	*Q. emoryi*	*Q. hypoleucoides*
1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.49±0.02	0.56±0.03
2	0.49±0.03	0.52±0.06	0.78±0.02	0.81±0.03	0.29±0.01	0.81±0.07	0.52±0.05	0.53±0.02	0.26±0.01	0.60±0.03	0.78±0.06	0.61±0.04
3	nd	nd	nd	nd	2.41±0.10	nd	nd	2.99±0.09	nd	nd	nd	nd
4	nd	nd	nd	nd	6.64±0.16	0.17±0.05	0.10±0.01	0.35±0.02	nd	0.30±0.02	1.86±0.15	1.64±0.07
5	3.52±0.29	6.83±0.18	7.35±0.32	5.18±0.22	1.18±0.03	12.17±1.29	5.35±0.35	4.97±0.19	3.38±0.14	5.21±0.29	nd	nd
6	4.87±0.10	1.78±0.05	1.73±0.05	5.01±0.19	4.43±0.09	3.89±0.09	3.31±0.16	4.64±0.15	2.29±0.08	5.85±0.19	nd	nd
7	2.10±0.18	6.85±0.13	4.90±0.16	2.22±0.08	12.95±0.19	4.23±0.07	3.43±0.15	1.90±0.05	4.38±0.33	1.65±0.08	0.24±0.02	1.55±0.06
8	1.27±0.04	0.02±0.01	0.05±0.00	1.27±0.03	nd	0.67±0.04	1.20±0.06	0.88±0.03	0.61±0.03	1.08±0.05	nd	nd
9	3.11±0.15	2.29±0.07	2.05±0.06	4.99±0.08	6.39±0.12	5.37±0.71	2.34±0.09	3.10±0.12	2.38±0.12	4.02±0.09	nd	nd
10	0.93±0.06	1.75±0.05	1.87±0.07	1.76±0.03	1.99±0.04	2.32±0.31	0.99±0.05	1.29±0.06	0.79±0.05	1.74±0.07	0.78±0.03	0.62±0.02
11	1.48±0.05	1.31±0.08	1.10±0.07	1.91±0.06	1.70±0.07	2.28±0.32	2.02±0.11	1.16±0.05	1.33±0.03	1.92±0.09	nd	nd
12	4.17±0.35	10.15±0.27	6.79±0.46	4.67±0.17	5.66±0.28	6.52±0.67	7.45±0.38	1.64±0.07	4.04±0.17	2.69±0.11	nd	nd
13	0.37±0.04	3.43±0.11	3.07±0.14	0.51±0.02	nd	1.22±0.14	0.93±0.08	0.09±0.02	0.40±0.03	0.23±0.02	nd	nd
14	nd	0.94±0.05	0.89±0.04	0.04±0.01	nd	0.86±0.04	nd	0.84±0.02	nd	0.56±0.02	nd	nd
15a	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.43±0.12	3.60±0.15
15b	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	8.64±0.34	8.08±0.25
16	0.41±0.03	1.41±0.07	1.25±0.03	1.26±0.02	6.17±0.15	1.95±0.35	0.49±0.05	0.30±0.04	0.38±0.01	0.74±0.03	nd	nd
17	0.58±0.03	1.18±0.07	1.20±0.05	1.44±0.04	nd	1.33±0.25	0.58±0.04	0.41±0.02	0.02±0.01	0.53±0.03	nd	nd
18	0.38±0.03	0.92±0.15	2.00±0.07	0.75±0.02	0.99±0.05	1.03±0.15	0.48±0.01	0.22±0.01	0.41±0.02	0.39±0.03	nd	nd
19	nd	0.82±0.06	0.70±0.06	nd	0.27±0.01	nd	nd	nd	nd	nd	nd	
20	0.64±0.02	0.91±0.05	1.17±0.03	1.18±0.03	0.42±0.01	1.52±0.22	0.87±0.04	0.72±0.03	0.54±0.02	0.73±0.04	nd	nd
21	0.32±0.03	0.41±0.01	0.35±0.02	0.09±0.01	0.58±0.02	0.44±0.09	0.61±0.03	0.15±0.01	0.38±0.02	0.18±0.01	nd	nd
22	0.52±0.04	1.37±0.06	1.29±0.04	1.88±0.03	0.46±0.02	2.71±0.53	0.55±0.03	0.47±0.02	0.33±0.02	0.93±0.04	1.42±0.08	1.65±0.08

Note: nd, not detected; concentration < 0.01 mg·g^{-1}.

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Most of the variation among white oaks in the $\log_e$+1 concentration of individual phenolics was attributable to variation in the $\log_e$+1 RCs. The ratio of the sum of the eigenvalues from discriminant analyses on the absolute and RCs indicates that approximately 59.21/75.27 = 78.7% of the total variation in phenolics among white oaks was due to variation in RCs between phenolics.

Size of foliar ellagitannins among white oaks

A MANOVA of the AC of ellagitannins in white oaks revealed ACs of ellagitannins to vary significantly across species ($p < 0.001$, Table 4). Univariate results indicate that 11 of the 12 ellagitannins significantly varied across white oaks ($p < 0.001$); only acutissimin A (**11**) was not significantly different across species ($p = 0.13$).

Variation among white oaks in ACs of ellagitannins produced four significant discriminant functions. The first three discriminant axes accounted for approximately 96% of the variation among species (Table 4, Fig. 3E). The first axis accounted for approximately 66% of the variation (Table 7) and represented decreasing concentrations of castavaloninic acid (**6**), vescalagin (**7**), and mongolinin A (**8**), as well as increasing concentrations of cocciferin D_2 (**12**) and ellagitannin **13**. The first discriminant axis of the ACs primarily separated QGR and QGA from the rest of the white oaks (Fig. 3E). The second axis accounted for around 25% of the variation and was delineated primarily by increasing concentrations of ellagitannin (**14**). The second discriminant axis separated QP, QMO and QT from the rest of the white oaks (Fig. 3E). The third discriminant axis explained an additional 4% of the variation and chiefly represented decreasing concentrations of all ellagitannins except vescalagin (**7**), vescavaloninic acid (**5**), and ellagitannin (**14**). The third axis served to separate QMO and QP from QT, and QR and QO from QA and QHA (Fig. 3E).

Shape of foliar ellagitannins among white oaks

MANOVA found the RCs of ellagitannins to vary significantly across white oak species ($p < 0.001$, Table 4). All 11 $\log_e$+1 RCs of ellagitannins significantly varied across white oaks ($p < 0.001$).

Variation among white oak species in RCs of ellagitannins produced four significant discriminant functions. The first three discriminant axes accounted for 97% of the variation among species (Table 4, Fig. 3F). The first axis described 72% of the variation (Table 7) and represented increasing RCs of vescalagin (**7**), cocciferin D_2 (**12**), and ellagitannin **13,** and decreasing RCs of castavaloninic acid (**6**), mongolinin A (**8**) and pedunculagin (**9**). The second discriminant axis accounted for approximately 19% of the variation and was delineated by increasing RCs of ellagitannin **14**, as well as decreasing RCs of cocciferin D_2 (**12**). The third discriminant axis explained an additional 5% of the variation and chiefly represented decreasing RCs of vescalagin (**7**) and vescavaloninic acid (**5**), as well as increasing relative amounts of castalagin (**10**). Similar to the ordination of the ACs of ellagitannins in white oaks, the first discriminant axis separated out QGA and QGR, the second discriminant axis distinguished QP, QMO and QT, while the third axis served to separate QMO, QP, QT, QR, QO, QA, and QHA (Fig. 3F).

Almost all of the variation among white oaks in the $\log_e$+1 concentrations of ellagitannins was attributable to variation in the $\log_e$+1 RCs. The ratio of the sum of the eigenvalues from discriminant analyses on the absolute and RCs indicates that approximately 23.49/25.78 = 91.1% of the total variation in ellagitannins among white oaks was due to variation in RCs of ellagitannins.

Discussion

There is complex variation in the production of individual foliar phenolics across oak species of the Chihuahuan Desert. Significant differences exist among oak species in both the absolute and relative concentration of phenolics. The variation of phenolics in multivariate space is rich and complex; phenolics do not vary in unison. This is true whether black oaks and white oaks are considered in the analysis or a single group of phenolics (ellagitannins) is used to ordinate oaks within a subgenus (*Quercus* sect. *Quercus*). Further, most of the overall variation in phenolic concentrations can be attributed to differences in the relative concentration of phenolics.

Across all oak species, a majority of the variation in the absolute and relative concentration of phenolics was attributed to differences between black oaks and white oaks. Much of the ellagitannin production in white oaks may be characterized as subsequent products of castalagin and vescalagin. In contrast, the black oaks also produce castalagin and vescalagin, but in relatively lower amounts (Table 3), and contain no detectable concentrations of castalagin or vescalagin derivatives. Black oak ellagitannins are dominated by the production of a large (molecular weight = 1724), isomeric ellagitannin that has yet to be identified structurally. The differences between black and white oak phenolic phenotypes may help explain profound ecological differences between the subgenera. For instance, the host-range of cynipid gall wasp species never spans across subgenera (Cornell 1985, 1996) and their host distribution correlates well with host phenolic chemistry (Abrahamson et al. 2003). Further, summary tannins have been correlated to gall wasp survival (Taper et al. 1986) in via suppression of parasite colonization (Taper and Case 1987). Therefore interspecific variation in resistance of host-plant phenolic phenotypes to endophytic microorganisms and parasites may well be important to determining the host range and survival of gall-forming arthropods. Second, recent studies of host range in the pathogen, *Phytophthora ramorum* (a.k.a. sudden oak death) in coastal oak forests of California, USA. have found white oaks to be resistant to infection while black oaks are suffering severely through an outbreak of the pathogen (Rizzo and Garbelotto 2003). While no known studies exist concerning the role of phenolics in *P. ramorum* resistance, interspecific variability in phenolic-based pathogen resistance has been shown in similar systems, such as *Eucalyptus* species (Cahill and McCombs 1992). Clearly, further study of subgenera-level differences in *Quercus* phenolic phenotypes and corresponding herbivore loads and pathogen susceptibility are of interest.

Surprisingly, a large portion of variation in the absolute and relative concentration of phenolics across all species could also be attributed to *Q. muhlenbergii*. This predomi-

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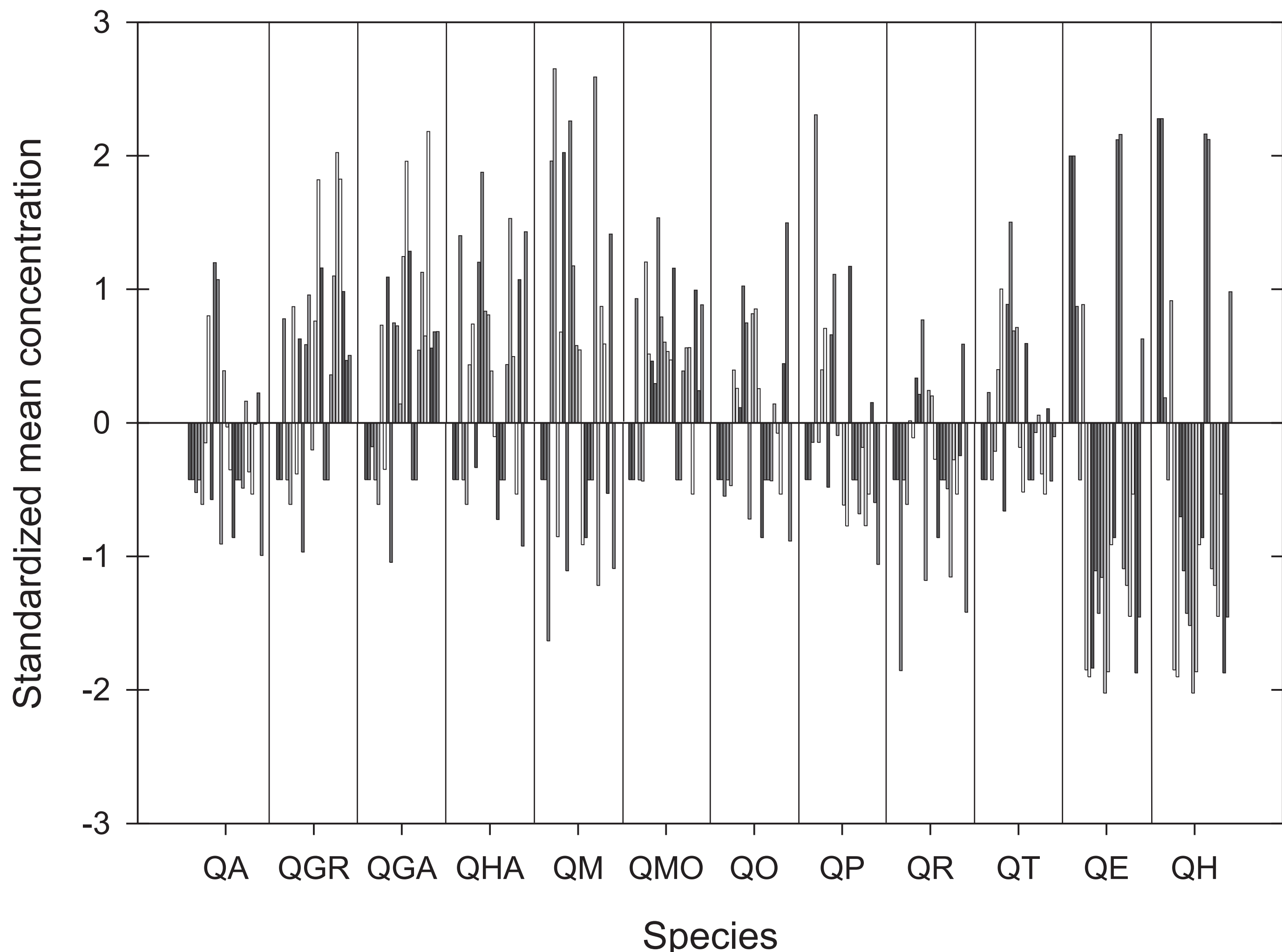
Fig. 2. Standardized mean concentrations of phenolics in oaks of the Chihuahuan Desert. Standardized mean concentrations are the mean concentrations for a given species, minus the grand mean of all species, divided by the standard deviation of species means. Standardized means are listed in order of the compound enumeration in Table 1; species abbreviations are those indicated in Table 1.



nantly eastern white oak, whose range into New Mexico represents its most westerly extent, was found to have a dissimilar composition of phenolic acids, hydrolysable tannins and flavonoid glycosides compared to the other Chihuahuan Desert white oaks. While the phenolic phenotype of *Q. muhlenbergii* is intermediate between the white and black oaks of the southwestern United States, the phenolic metabolism of *Q. muhlenbergii* appears to be quite similar to the white oaks of the eastern United States (J.-P. Salminen, unpublished data).

Size and shape analysis provides a powerful conceptual and analytical framework that is sensitive to variation in phytochemistry on absolute and relative scales, and to the co-variation of plant phytochemistry and ecological factors. This has great utility for understanding the metabolism of plant phenolics. In the case of Chihuahuan Desert white oaks, size and shape analysis revealed important aspects of interspecific variation in ellagitannin biosynthesis. For instance, comparison of the second discriminant axes of the absolute and relative concentrations of ellagitannins revealed that the relative amounts of the end-products mongolinin A (**8**), acutissimin A (**11**), and cocciferin D_2 (**12**) (Fig. 1) were produced in higher amounts in *Q. oblongifolia*, *Q. rugosa*, *Q. arizonica*, and *Q. havardii*. Consistent with the biosynthesis of oak ellagitannins, an increase in the relative concentration of precursors such as vescavaloninic acid (**5**) and other end-products like castavaloninic acid (**6**) was found in the remaining white oaks. A simple examination of the absolute concentrations of these same compounds would not achieve the same result (Table 3). It is also useful to note that size and shape analysis is flexible and may be extended to include such factors as seasonal components of phytochemistry (e.g., Osier et al. 2000; Riipi et al. 2004; also see Boecklen et al. 1991) that may further elucidate variation in the biosynthesis of plant phenolics (Salminen et al. 2004).

The results of this study suggest a strong genetic component in the production of foliar phenolics between species. First, there is little evidence for abiotic determinants of phenolic phenotypes across species. For example, in the southwestern United States, *Q. muhlenbergii* occupies mesic canyon slopes and riparian edges to 2200 m, the same habitat type occupied by *Q. gambelii*. If genera-level production of phenolics were predominantly determined through ecology, *Q. muhlenbergii* and *Q. gambelii* populations would be

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Table 4. Results of MANOVA and discriminant function analysis (DFA) for absolute (size) and relative (shape) concentrations of phenolics across white and black oak species, phenolics in white oak species only (excluding *Q. muhlenbergii*), and ellagitannins in white oak species only (excluding *Q. muhlenbergii*).

	MANOVA				DFA					
					Eigenvalue			Cumulative variation		
Concentration	Statistic	Value	Approx. *F*	df	DF1	DF2	DF3	DF1	DF2	DF3
Phenolics: white and black oaks										
Absolute	Wilks' Lambda	0.000	20.445	253, 825	105.38	39.95	21.76	53.6	73.9	85.0
	Pillai Trace	7.033	6.936	253, 990						
	Hotelling–Lawley Trace	196.593	60.751	253, 860						
Relative	Wilks' Lambda	0.000	21.285	242, 828	100.02	39.5	21.8	52.9	73.8	85.3
	Pillai Trace	6.974	7.166	242, 1001						
	Hotelling–Lawley Trace	189.062	61.861	242, 871						
Phenolics: white oaks (excluding *Q. muhlenbergii*)										
Absolute	Wilks' Lambda	0.000	13.410	160, 447	38.59	23.57	7.34	49.9	80.3	89.8
	Pillai Trace	5.386	6.698	160, 520						
	Hotelling–Lawley Trace	77.388	27.270	160, 450						
Relative	Wilks' Lambda	0.000	13.018	152, 451	24.95	21.66	7.34	40.7	76.0	88.0
	Pillai Trace	5.330	6.936	152, 528						
	Hotelling–Lawley Trace	61.302	23.089	152, 458						
Ellagitannins: white oaks (excluding *Q. muhlenbergii*)										
Absolute	Wilks' Lambda	0.001	10.022	80, 439	17.57	6.67	1.13	66.7	92.0	96.3
	Pillai Trace	3.118	4.790	80, 600						
	Hotelling–Lawley Trace	26.347	21.819	80, 530						
Relative	Wilks' Lambda	0.002	10.471	72, 427	17.31	4.47	1.32	72.3	91.0	96.5
	Pillai Trace	2.990	5.039	72, 608						
	Hotelling–Lawley Trace	23.929	22.350	72, 538						

Note: Analyses were performed on the $\log_e$+1 concentration size and $\log_e$+1 concentration – $\log_e$+1 geometric mean shape variables.

expected to contain similar phenolic phenotypes; this was not the case. Also, locality of species collection was not obviously related to phenolic phenotypes (Table 1). Importantly, hybridization is a common phenomenon among oaks and the likelihood of introgression of phenolic traits between oak species is high. The range of *Q. muhlenbergii* extends from Maine to southeastern New Mexico; *Q. muhlenbergii* occurs in contact with much different oaks than those found in the Chihuahuan Desert across most of its range. Overall, there is limited geographical contact between *Q. muhlenbergii* and the oaks represented in this study, and putative hybrids of *Q. muhlenbergii* and *Q. mohriana* have not been found during our research. Therefore, the vast majority of introgressive gene flow into *Q. muhlenbergii* is likely to come from oaks of the eastern and midwestern United States. The striking similarities between phenolic phenotypes in *Q. gambelii* and *Q. grisea* may also be explained by hybridization. These trees were collected from a well-studied hybrid zone, but had been previously genotyped as pure *Q. gambelii* and *Q. grisea* (Howard et al. 1997). Despite their genetic identity based upon population genetic markers, these species were found to produce highly correlated phenolic phenotypes. Moreover, because hybridization is restricted to subgenera (Tucker 1959; Cottam et al. 1982), barriers to hybridization would also explain the significant differences in phenolic phenotypes between black oaks and white oaks. Strong genetic control of phenolic production would also be consistent with detailed genetic studies in other tree species (Keinänen et al. 1999; Klaper et al. 2001; Osier and Lindroth 2001; Laitinen et al. 2005).

This study was necessarily limited to only a short timeframe and did not examine geographic variation in oak leaf phenolics, thereby limiting information about ecological determinants of phenolic production. Moreover, we also do not suggest that genetics explain the majority of variation in phytochemical phenotypes. Quite to the contrary, it is well-known that within species the production of phenolics strongly varies both seasonally (e.g., Salminen et al. 2001) and environmentally (e.g., Moore et al. 2004). Therefore, geographic and temporal variation in phenolic phenotypes within species likely represents environmentally induced fluctuation within a genetic template. Noting that these oak species were so highly variable at a single time period during the growing season, the addition of seasonal and geographic effects would surely balloon the observed variation in phenolics in oaks with complex effects for associated organisms. These studies may be best pursued through the use of abiotic mosaics which have been great utility for the investigation of co-variation between phytochemical characteristics and numerous ecological factors, as demonstrated in other systems (Hall et al. 2005*a*, 2005*b*; Yarnes and Boecklen 2005, 2006) and will be a useful tool in further studies of oak species phenolic phenotypes.

Most reports of ecological and genetic variation in phenolic production have focused on summary phenolics and contain little information about the variation in the absolute

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Fig. 3. Phenolic composition of oak species in discriminant space. Location is represented by the centroid of each species along the corresponding discriminant function. Labeling abbreviations of species follow thoseoutlined in Table 1. (A) Absolute concentrations for all oaks. (B) Relative concentrations forall oaks. (C) Absolute concentrations for white oaks. (D) Relative concentrations for white oaks. (E) Absolute concentrations for ellagitannins in white oaks. (F) Relative concentrations for ellagitannins in white oaks.

Table 5. Correlation coefficients (*r*) between $\log_e$+1 transformed absolute and relative concentrations of phenolic compounds in Chihuahuan Desert oak species and the first three canonical axes from their respective discriminant function analysis.

	Absolute			Relative		
Compound	DF1	DF2	DF3	DF1	DF2	DF3
1	**–0.875**	0.276	–0.015	**–0.799**	0.360	–0.178
2	–0.026	0.245	0.035			
3	–0.019	**–0.735**	–0.424	–0.210	**–0.562**	**–0.522**
4	**–0.633**	**–0.671**	–0.114	**–0.760**	**–0.541**	–0.199
5	0.833	0.075	0.170	0.839	0.227	0.133
6	0.794	–0.301	–0.356	0.737	–0.200	–0.512
7	0.368	**–0.668**	0.442	0.256	–0.664	0.429
8	0.599	0.279	**–0.597**	0.266	0.460	**–0.704**
9	0.731	–0.453	–0.159	0.735	–0.422	–0.319
10	0.356	–0.290	0.196	0.043	–0.091	0.053
11	0.709	–0.219	–0.091	–0.463	**0.528**	–0.176
12	0.717	–0.255	0.401	0.718	–0.191	0.409
13	0.435	0.148	**0.774**	0.276	0.362	**0.821**
14	0.405	0.055	0.287	0.079	0.354	0.146
15a	**–0.931**	0.296	–0.017	**–0.913**	0.326	–0.093
15b	**–0.943**	0.301	–0.018	**–0.932**	0.316	–0.073
16	0.264	**–0.730**	0.261	0.100	**–0.775**	0.203
17	0.551	0.223	0.282	0.341	**0.550**	0.190
18	0.451	–0.257	0.470	0.215	–0.066	0.457
19	0.133	–0.243	**0.755**	–0.312	0.075	**0.504**
20	0.724	0.015	0.156	0.607	0.420	–0.015
21	0.364	–0.363	0.249	–0.190	–0.019	–0.013
22	–0.148	0.295	0.257	–0.462	**0.502**	0.124

Note: Compounds with coefficients given in bold contribute strongly to the ordination of oak phenotypes in discriminant space.

and relative production of individual phenolics. Phytochemical phenotypes of plants are far more complex than the variation accounted for by summary measures and this must be taken into consideration by ecologists. Further, plants contain immense variation in their responses to biotic (e.g., microbes, herbivores, symbionts) and abiotic factors (e.g., light, inorganic resources, CO_2). Failure to account for components of phytochemical variation and their relationship with complex ecological factors may radically delay the development of general theories regarding the role of phytochemistry and putative defense compounds, such as phenolics, in plant ecology.

In summary, we have demonstrated the utility of size and shape analysis in the investigation of phenolic phenotypes in oak species. While nearly all phenolics significantly vary across taxa, standard multivariate results are unable to identify those compounds most important to interspecific variation in the absolute and relative concentrations of phenolics in oaks. However, size and shape analysis demonstrates that phenolics do vary in a complex manner, both at absolute and relative scales, across oak species of the Chihuahuan Desert. Strong allometric variation in phenolic concentrations was evident when considering different combinations of oak species, or ellagitannins or all phenolics. In each case, the primary source of variation was attributed to differences in the relative concentrations of phenolics, that is , their shape. We

Table 6. Correlation coefficients (*r*) between $\log_e$+1 transformed absolute and relative concentrations of phenolic compounds in white oak species (excluding *Q. muhlenbergii*) and the first three canonical axes from their respective discriminant function analysis.

	Absolute			Relative		
Compound	DF1	DF2	DF3	DF1	DF2	DF3
2	0.029	0.059	0.356			
3	**–0.927**	**–0.359**	0.054	**–0.907**	–0.276	–0.074
4	–0.343	**–0.362**	0.180	–0.648	–0.278	–0.180
5	–0.060	0.314	0.284	0.119	–0.214	0.216
6	0.063	**–0.690**	0.337	–0.549	**0.619**	0.163
7	0.011	0.702	–0.180	0.443	–0.416	**–0.357**
8	0.192	**–0.816**	–0.015	–0.518	**0.694**	–0.178
9	0.158	**–0.345**	**0.436**	–0.367	**0.613**	0.336
10	0.001	0.251	**0.499**	0.035	–0.012	**0.499**
11	0.246	**–0.206**	0.027	–0.167	0.562	–0.205
12	0.261	0.575	–0.147	0.622	–0.169	**–0.324**
13	0.054	0.884	0.026	0.720	**–0.594**	–0.118
14	**–0.479**	0.431	**0.485**	–0.174	**–0.483**	0.302
16	0.216	0.429	**0.396**	0.433	–0.015	0.306
17	0.097	0.345	**0.410**	0.220	–0.070	0.329
18	0.114	0.553	0.182	0.408	–0.205	–0.007
19	–0.121	0.824	0.071	0.293	–0.424	–0.169
20	0.054	0.215	0.252	–0.043	0.123	–0.001
21	0.106	0.254	**–0.442**	–0.004	0.162	**–0.779**
22	0.187	0.294	**0.506**	0.281	0.070	**0.489**

Note: Compounds with coefficients given in bold contribute strongly to the ordination of oak phenotypes in discriminant space.

Table 7. Correlation coefficients (*r*) between $\log_e$+1 transformed absolute and relative concentrations of ellagitannins in white oak species (excluding *Q. muhlenbergii*) and the first three canonical axes from their respective discriminant function analysis.

	Absolute			Relative		
Compound	DF1	DF2	DF3	DF1	DF2	DF3
5	0.300	0.321	**0.160**	0.280	0.340	**–0.341**
6	**–0.736**	0.185	–0.210	**–0.858**	0.139	0.146
7	**0.735**	–0.077	**0.299**	**0.694**	–0.220	**–0.339**
8	**–0.828**	–0.235	–0.096	**–0.830**	–0.247	0.038
9	–0.418	0.170	–0.145	**–0.792**	0.140	0.137
10	0.181	0.383	–0.259	–0.025	0.484	**0.292**
11	–0.220	–0.152	–0.050			
12	**0.575**	–0.274	–0.076	**0.615**	**–0.509**	0.101
13	**0.869**	0.028	–0.194	**0.940**	–0.083	0.201
14	0.421	**0.826**	**0.132**	0.241	**0.738**	**–0.219**

Note: Compounds with coefficients given in bold contribute strongly to the ordination of oak phenotypes in discriminant space.

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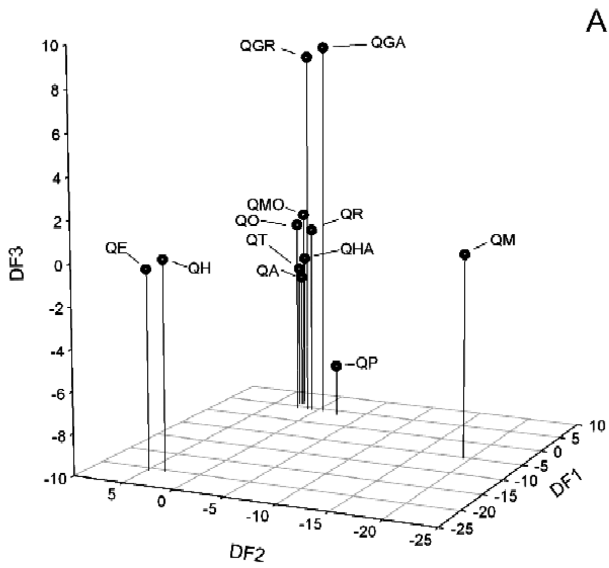

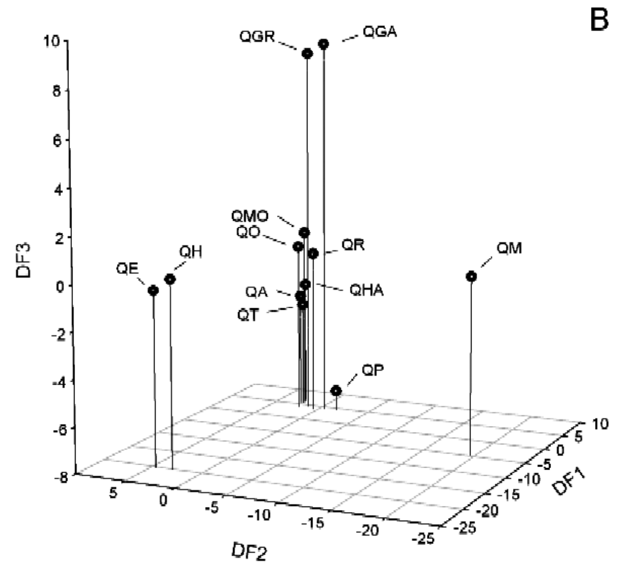

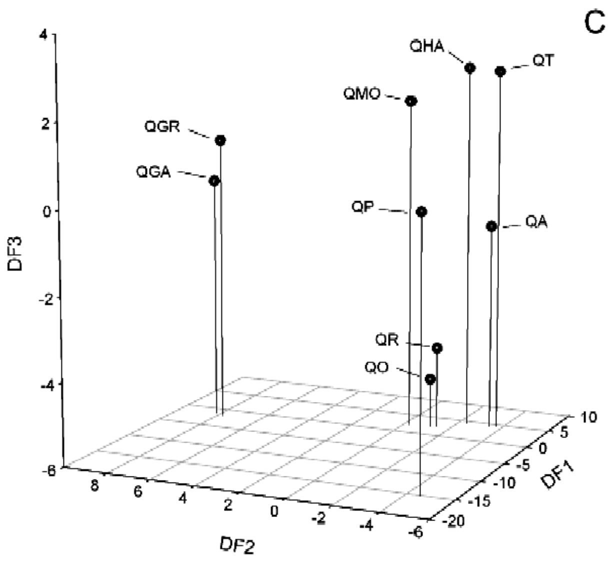

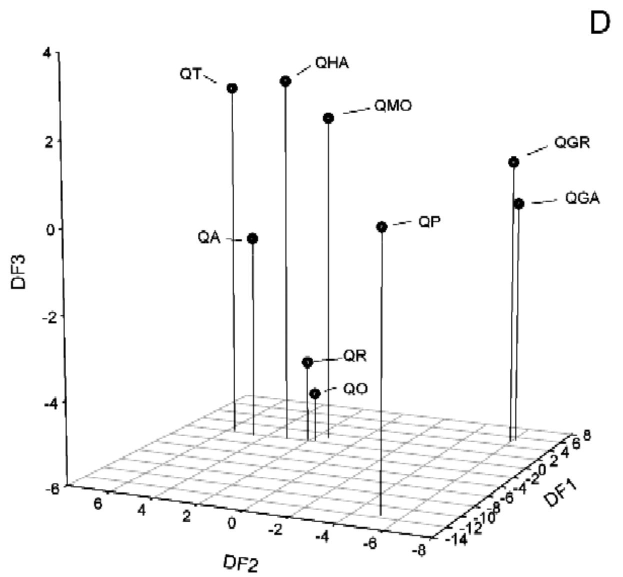

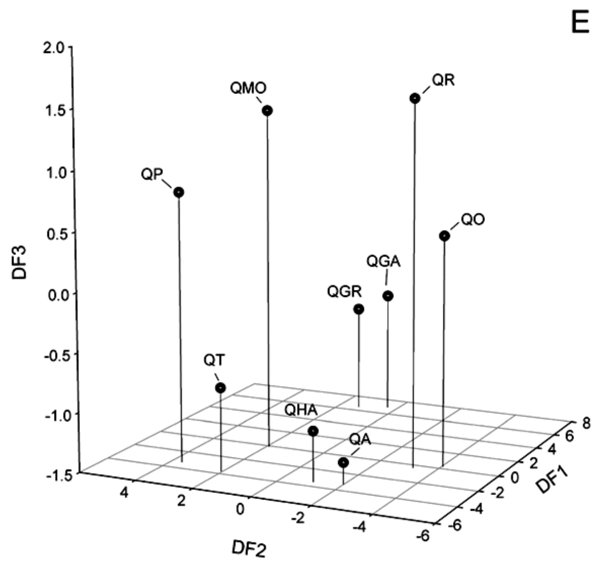

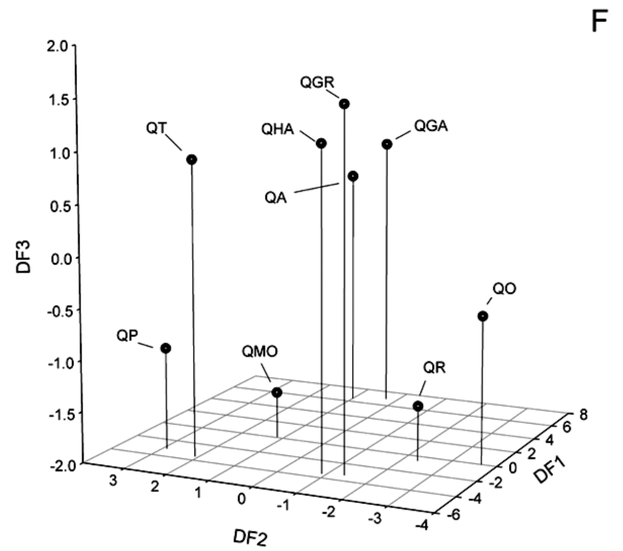

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also reported the occurrence of complex tannins in oaks of North America for the first time, we updated the proposed pathway of ellagitannin biosynthesis in oaks, and we provided a comprehensive survey of individual phenolics in oaks of the southwestern United States.

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