

Assessing the influence of biogeographical region and phylogenetic history on chemical defences and herbivory in *Quercus* species



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ABSTRACT

Biogeographical factors and phylogenetic history are key determinants of inter-specific variation in plant defences. However, few studies have conducted broad-scale geographical comparisons of plant defences while controlling for phylogenetic relationships, and, in doing so, none have separated constitutive from induced defences. This gap has limited our understanding of how historical or large-scale processes mediate biogeographical patterns in plant defences since these may be contingent upon shared evolutionary history and phylogenetic constraints. We conducted a phylogenetically-controlled experiment testing for differences in constitutive leaf chemical defences and their inducibility between Palearctic and Nearctic oak species (*Quercus*, total 18 species). We induced defences in one-year old plants by inflicting damage by gypsy moth larvae (*Lymantria dispar*), estimated the amount of leaf area consumed, and quantified various groups of phenolic compounds. There was no detectable phylogenetic signal for constitutive or induced levels of most defensive traits except for constitutive condensed tannins, as well as no phylogenetic signal in leaf herbivory. We did, however, find marked differences in defence levels between oak species from each region: Palearctic species had higher levels of constitutive condensed tannins, but less constitutive lignins and less constitutive and induced hydrolysable tannins compared with Nearctic species. Additionally, Palearctic species had lower levels of leaf damage compared with Nearctic species. These differences in leaf damage, lignins and hydrolysable (but not condensed) tannins were lost after accounting for phylogeny, suggesting that geographical structuring of phylogenetic relationships mediated biogeographical differences in defences and herbivore resistance. Together, these findings suggest that historical processes and large-scale drivers have shaped differences in allocation to constitutive defences (and in turn resistance) between Palearctic and Nearctic oaks. Moreover, although evidence of phylogenetic conservatism in the studied traits is rather weak, shared evolutionary history appears to mediate some of these biogeographical patterns in allocation to chemical defences.

1. Introduction

The interactions between plants and phytophagous insects are more than 350 million years old (Labandeira, 2007), and have resulted in co-evolutionary arms races involving strong selection for increased plant defence and herbivore counter-defence (Ehrlich and Raven, 1964; Becerra, 1997; Berenbaum and Zangerl, 1998; Futuyama and Agrawal, 2009). The plant defensive arsenal against phytophagous insects includes a broad repertoire of structural barriers (e.g., spines, thorns, trichomes) and chemical compounds (e.g., phenolics, alkaloids, terpenoids) that kill, repel or reduce the performance of herbivores

(Agrawal, 2007; Núñez-Farfán et al., 2007). These physical and chemical traits exhibit enormous variation among plant species, even within a single genus (Agrawal, 2011), and elucidating the mechanisms and processes that originate and maintain such diversity represents a central challenge in evolutionary ecology (Becerra, 1997; Berenbaum and Zangerl, 1998; Agrawal, 2007, 2011).

The influence of historical processes on the evolution of plant defences can be inferred from analysing differences in allocation patterns among plant taxa belonging to different biogeographical regions (Bryant et al., 1994; Marquis et al., 2012; Ricklefs and He, 2016; Grutters et al., 2017). In cases where geographical differences have

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been observed, further investigations based on historical data and current observations on broad-scale drivers have served to point at the most probable processes or factors underlying contrasting trajectories of plant defence evolution (Bryant et al., 1994; Steinberg et al., 1995; Desurmont et al., 2011; Craft et al., 2013; Carrillo-Gavilán et al., 2015; Grutters et al., 2017). For example, comparisons of Nearctic (i.e., North America) and Palearctic (i.e., northern Eurasia) plant taxa have demonstrated that current differences in plant defensive investment between these regions have resulted from processes such as higher extinction rates and lower rates of recolonization of plant and herbivore species during the last glacial period in Palearctic regions compared with Nearctic regions (Archetti et al., 2009; Lev-Yadun and Holopainen, 2009).

In recent decades, with the advent of molecular techniques and phylogenetic comparative analyses, ecologists have been able to address the influence of phylogenetic non-independence on patterns of inter-specific variation in plant defence allocation (Becerra, 1997; Agrawal et al., 2009a; Kursar et al., 2009; Desurmont et al., 2011; Pearse and Hipp, 2012). Ehrlich and Raven (1964) predicted that more closely related plant species should share similar defensive chemistry, that closely related herbivores should feed on closely related plants, and that co-evolutionary arms races should lead to increased levels of plant defence and herbivore counter-defences (Agrawal et al., 2009a,b,c; Pearse and Hipp, 2009; Craft et al., 2013). Nonetheless, some studies have reported weak or lacking phylogenetic signals in plant defensive traits (e.g., Kursar et al., 2009; Rasmann and Agrawal, 2011; Endara et al., 2017), questioning the influence of shared evolutionary history on patterns of inter-specific variation in plant defences as proposed by classic theory. This latter group of studies suggests alternative processes influencing macro-evolutionary patterns in plant defence such as resource-tracking by herbivores following plant diversification (Endara et al., 2017) or convergent evolution (Kursar and Coley, 2003; Agrawal, 2007).

To address interspecific variation in plant defences, one key aspect is distinguishing between constitutive (i.e., basal levels expressed at any given time) and induced (i.e., those activated, increased, or synthesized only following herbivory) levels of defensive traits (Karban, 2011). Each strategy is favored over the other depending on the ecological context in which plants are embedded and studies have found that these defensive strategies in some cases trade off, i.e. increases in one strategy frequently lead to reductions in the other (Rasmann et al., 2011; Moreira et al., 2014). Although previous work has analysed these two modes of plant defence within a macro-evolutionary framework (e.g., Kempel et al., 2011; Rasmann and Agrawal, 2011; Moreira et al., 2014; Pellissier et al., 2016), few studies have compared allocation to constitutive and induced defensive strategies among geographically isolated taxa at the regional or continental level (but see Carrillo-Gavilán et al., 2015), therefore limiting our understanding of how historical process have contributed to shape plant defence evolution. In conducting these broad-scale comparisons, a key aspect to consider are the evolutionary relationships among the studied taxa; groups of closely related species may exhibit more similar patterns of allocation and such species could be geographically constrained to a given region (e.g., Fabaceae; Seigler et al., 1989). Accordingly, assessing the influence of evolutionary relationships while conducting biogeographical comparisons represents a robust approach for understanding how historical processes shape large-scale patterns of inter-specific variation in plant defensive traits (Agrawal et al., 2009a; Pearse and Hipp, 2012).

In this study, we assessed the production of chemical defences in oak species (*Quercus* spp.) from two disjointed biogeographical regions while controlling for the evolutionary relationships among these species. Phylogenetic and paleobotanical data suggest that the center of diversification for *Quercus* spp. is at middle latitudes of America; some species subsequently migrated to the Old World prior to the break-up of land bridges linking the northern continents, whereas others (e.g., red oaks) evolved too late to cross (Nixon, 1993; Manos et al., 1999). In

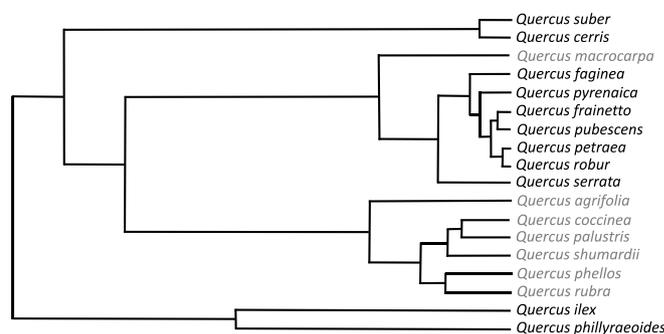


Fig. 1. Phylogenetic tree of the studied *Quercus* species based on Single Nucleotide Polymorphism matrices by ddRAD sequencing. Nearctic species are shown in grey font and Palearctic species are shown in black font.

addition, previous studies have held the existence of lower extinction rates of temperate tree species and higher rates of recolonization of plant and herbivore species since the last glacial period in Nearctic regions compared with Palearctic ones (Archetti et al., 2009; Lev-Yadun and Holopainen, 2009). According to these studies, Nearctic oak species would be better chemically defended than Palearctic ones. To test this, we performed a greenhouse experiment using one-year-old plants belonging to 18 oak species, 11 from the Palearctic region and seven from the Nearctic region (Fig. 1), and quantified the concentration of constitutive phenolic compounds in leaves as well as their induced levels. Defence induction was measured after damage inflicted by gypsy moth larvae (*Lymantria dispar*, Lepidoptera), a generalist herbivore that is native to the Old World but was introduced in North America and feeds on leaves of oak species from both biogeographical regions. After inducing plants, we also estimated the amount of leaf area consumed by the larvae to assess herbivore resistance in these species. In doing so, we sought to (i) assess the influence of shared evolutionary history (i.e., relatedness) on patterns of inter-specific variation chemical defences and herbivory by estimating the phylogenetic signal in these traits for the studied oak species, and (ii) test for differences between Nearctic and Palearctic oak species in allocation patterns to constitutive and induced chemical defences and herbivore resistance. In addition, we determined whether any such difference between regions is influenced by the shared evolutionary history of the studied species. This may occur if closely related basal vs. more derived species differ in their patterns of defence allocation and are represented differently in each biogeographical region. Overall, this study provides a unique assessment of the independent influences of long-term isolation (accounted for biogeographical region) and genetic relatedness (modelled by shared evolutionary history) on patterns of inter-specific variation in oak defences.

2. Results

2.1. Phylogenetic signal in plant chemical defences and herbivory

We found a significant phylogenetic signal for the concentration of constitutive condensed tannins, but not for any of the other classes of phenolic compounds (Table 1a). Likewise, we found no evidence of a phylogenetic signal in the inducibility for any of group of phenolic compounds (Table 1b) or in the amount of leaf herbivory (Table 1c).

2.2. Effects of biogeographical region and shared evolutionary history on leaf defences

Oak species significantly differed in their concentrations of constitutive leaf lignins and condensed and hydrolysable tannins, but not flavonoids (Fig. SMI in the Supplementary Material). Statistical models indicated a significant effect of biogeographical region on constitutive

Table 1

Analysis of the phylogenetic signal ($\lambda = \text{lambda}$) in (a) constitutive allocation to leaf chemical defences (flavonoids, lignins, condensed tannins and hydrolysable tannins), (b) inducibility of these defences by gypsy moth *Lymantria dispar* larvae and (c) leaf herbivory by gypsy moth in 18 *Quercus* species. The maximum-likelihood estimate of λ (an estimate of the phylogenetic signal) and its associated log-likelihood values ('ln lik') are shown, as well as when λ is forced to $\lambda = 0$. λ varies between 0 and 1. A λ value of 1 indicates phylogenetic conservatism consistent with the tree topology and a random walk model (i.e., herbivory or defence similarity is directly proportional to the extent of shared evolutionary history), whereas a λ value of 0 indicates no influence of shared ancestry on herbivory or defence (i.e., phylogenetic independence). Cases where the λ value differed significantly from 0 according to the log-likelihood ratio test are shown in bold type.

	λ_x	ln lik ($\lambda = x$)	ln lik ($\lambda = 0$)	P-value
(a) Constitutive defences				
Flavonoids	0	−32.75	−32.75	0.51
Lignins	0.3	−47.63	−48.16	0.30
Condensed tannins	0.7	−20.09	−22.79	0.004
Hydrolysable tannins	0	−100.34	−100.34	0.49
(b) Inducibility of defences				
Flavonoids	0	−25.03	−25.03	0.50
Lignins	0	−38.30	−38.30	0.62
Condensed tannins	0	−20.17	−20.17	0.33
Hydrolysable tannins	0	−91.35	−91.35	0.32
(c) Leaf herbivory				
	0	−37.11	−37.11	0.13

levels of lignins, condensed tannins, and hydrolysable tannins, but not flavonoids (Table 2a). Concentrations of constitutive lignins and hydrolysable tannins in leaves of Nearctic species were 118% and 125% greater, respectively, than those present in Palaearctic species (Fig. 2b and d), whereas the concentration of constitutive condensed tannins was 86% greater in Palaearctic species than in Nearctic ones (Fig. 2c). Follow-up analyses indicated that the effect of biogeographical region on constitutive lignins and hydrolysable tannins turned non-significant after including the effect of evolutionary history, with significant Δ DIC values observed in both cases for the model with vs. without this factor, whereas for constitutive condensed tannins the effect of region remained significant and the Δ DIC value was not significant (Table 2a). In the case of constitutive flavonoids, the Δ DIC value after including evolutionary history in the model was also non-significant (Table 2a).

Oak species also significantly differed in their inducibility of leaf lignins and condensed and hydrolysable tannins, but not flavonoids (Fig. SM2 in the Supplementary Material). There was a significant effect of biogeographical region on the inducibility of hydrolysable tannins, but not on the inducibility of flavonoids, lignins, or condensed tannins (Table 2b, Fig. 3a–d). In the first case, however, we found induced susceptibility in response to damage (i.e., levels of hydrolysable tannins after insect attack were lower than constitutive levels), and this reduction of defences was on average four-fold greater for Nearctic than for Palaearctic species (Fig. 3d, Fig. SM2). The effect of biogeographical region on the inducibility of hydrolysable tannins turned non-significant after accounting for evolutionary history, with a significant Δ DIC value with vs. without this factor (Table 2b).

2.3. Effects of biogeographical region and shared evolutionary history on leaf herbivory

Oak species significantly differed in the amount of leaf herbivory by gypsy moth larvae (Fig. SM3 in the Supplementary Material). Biogeographical region significantly predicted leaf herbivory (Table 2c), with Nearctic species exhibiting 84% greater mean value of leaf damage than Palaearctic species (Fig. 4). In addition, the effect of region turned non-significant after including the effect of evolutionary history in the model and the Δ DIC value was significant (Table 2c). Analyses testing

for associations between levels of leaf damage and constitutive defences across the studied species indicated that constitutive levels of lignins and hydrolysable tannins were positively correlated with leaf herbivory, whereas concentration of condensed tannins was negatively (marginally) correlated with herbivory (Table SM1 in the Supplementary Material).

3. Discussion

Our results showed that for most of the studied defensive traits, except constitutive condensed tannins, there was no detectable phylogenetic signal, suggesting that shared evolutionary history does not play an important role in shaping patterns of inter-specific variation in these groups of phenolic compounds for the studied oak species. We did, however, find a marked difference in constitutive levels of leaf chemical defences between Nearctic and Palaearctic species, particularly for lignins, condensed tannins, and hydrolysable tannins, but no difference between regions in the inducibility of most of these compounds (except hydrolysable tannins). Interestingly, Palaearctic species have higher levels of constitutive condensed tannins but less lignins and hydrolysable tannins compared with Nearctic species. In addition, except for constitutive condensed tannins, the effect of biogeographical region was lost after including the effect of evolutionary relatedness in the model, indicating an influence of phylogenetic relationships in shaping biogeographical differences in these defensive traits. We also found that Palaearctic species exhibited a lower mean level of leaf damage compared with Nearctic species suggesting higher resistance. Together, these findings suggest that large-scale historical processes have shaped differences in levels of constitutive defences (and in turn resistance) between biogeographical regions, and although evidence of phylogenetic conservatism in these defensive traits is rather weak, evolutionary history appears to mediate in some cases geographical differences in oak defence investment.

3.1. Weak phylogenetic signal in plant defence and herbivory

Inter-specific variation in constitutive levels of defensive traits levels and in their inducibility was not strongly determined by evolutionary relatedness. The only exception to this overall pattern was the significant phylogenetic signal detected on constitutive levels of condensed tannins. Likewise, we also found no evidence of a phylogenetic signal on herbivory, suggesting that weak phylogenetic structure in plant defences drove a concomitant pattern of resistance to herbivory that was independent of phylogenetic relationships. Previous studies have similarly reported low to moderate phylogenetic signals in constitutive and induced defences for species of the genus *Inga* (Kursar et al., 2009; Endara et al., 2017), *Bursera* (Becerra, 1997), *Asclepias* (Rasmann and Agrawal, 2011), *Pinus* (Carrillo-Gavilán et al., 2015), *Piper* (Salazar et al., 2016), *Solanum* (Haak et al., 2014) and *Oenothera* (Johnson et al., 2014). These results are inconsistent with co-evolutionary arms race theory which predicts concomitant phylogenetic structuring of plant defence investment and herbivore traits (Ehrlich and Raven, 1964; Futuyama and Agrawal, 2009), a pattern that has been supported by previous work reporting a strong phylogenetic signal in plant constitutive (Agrawal et al., 2009a,b; Armbruster et al., 2009; Pearse and Hipp, 2009; Desurmont et al., 2011; Pearse and Hipp, 2012) and induced (Kempel et al., 2011) defences. The fact that closely related oak species were not more similar in their patterns of allocation to chemical defences suggests that investment in these traits is evolutionarily labile and may depend more on rapid responses to local ecological conditions (e.g., climate, biotic interactions, resource availability) in each species' range. We do note, however, that the number of species analysed was relatively low and this could have influenced our ability to detect phylogenetic signals in the studied traits. Running further analyses for a greater number of oak species (as well as additional defensive traits) would be necessary to corroborate our findings and

Table 2

MCMCglmm models. Shown are the effects of biogeography (Palearctic vs. Nearctic) and phylogeny on (a) constitutive allocation to leaf chemical defences (flavonoids, lignins, condensed tannins and hydrolysable tannins), (b) inducibility of these defences by gypsy moth *Lymantria dispar* larvae and (c) leaf herbivory by gypsy moth in 18 *Quercus* species. For each chemical defence type and herbivory, we first ran a full model including biogeographical regions, plant height and damage (for inducibility data) as a fixed factors, and blocks as random factor (marked with an asterisks in the Table) (without phylogeny, “no P”). Subsequently, we ran a second model as above but including the phylogenetic variance-covariance matrix as a random factor to assess the influence of evolutionary history on biogeographical differences in defence production and herbivory (with phylogeny, “P”). Assuming Brownian motion, the expected covariance is directly proportional to the amount of shared evolutionary history. For each defensive trait and herbivory, we compared models with and without phylogeny using the Deviation Information Criterion (DIC), where a significantly smaller DIC value for the model with this factor ($\Delta\text{DIC} > 5$ without vs. with evolutionary history in the model) would indicate that evolutionary history significantly influences defences or herbivory. Significances between DIC values were assessed using Likelihood-ratio Tests (pLLR-test). In addition, if significant differences between biogeographical regions in defence expression or herbivory are shaped by shared evolutionary history of the studied taxa, then a significant effect of biogeographical region in the first model should turn non-significant after accounting for this factor. Significant *P*-values ($P < 0.05$) are typed in bold.

Variable	Source	Posterior mean	l-95% ci ^a	u-95% ci	pMCMC ^b	DIC	ΔDIC	pLLR-test (phylogeny effect)
(a) Constitutive defences								
Condensed tannins	Intercept (no P)	1.78	0.73	2.86	0.002			
	Intercept (P)	1.78	0.56	2.77	0.002			
	Biogeography (no P)	1.17	0.53	1.85	< 0.001			
	Biogeography (P)	1.17	0.53	1.87	< 0.001			
	Height (no P)	−0.01	−0.03	0.01	0.40			
	Height (P)	−0.01	−0.03	0.01	0.40			
	Block (no P)*	0.04	0	0.23				
	Block (P)*	0.02	0	0.1				
	No Phylogeny*					411.12		
	Phylogeny*	0	0	0		410.70	0.27	1
Hydrolysable tannins	Intercept (no P)	122.12	81.14	161.86	0.001			
	Intercept (P)	79.30	−141.99	297.52	0.43			
	Biogeography (no P)	−35.56	−61.14	−7.24	0.02			
	Biogeography (P)	−4.50	−179.41	195.24	0.94			
	Height (no P)	−1.07	−2.01	−0.27	0.01			
	Height (P)	−0.56	−1.53	0.39	0.26			
	Block (no P)*	11.9	0	20.17				
	Block (P)*	0	0	0				
	No Phylogeny*					1176.11		
	Phylogeny*	26547	7826	51434		1116.75	59.83	< 0.001
Lignins	Intercept (no P)	6.07	3.40	8.71	< 0.001			
	Intercept (P)	3.44	−7.07	14.68	0.51			
	Biogeography (no P)	−3.62	−5.46	−2.08	< 0.001			
	Biogeography (P)	−1.51	−12.33	6.98	0.68			
	Height (no P)	0.02	−0.03	0.08	0.52			
	Height (P)	0.02	−0.05	0.09	0.44			
	Block (no P)*	0.07	0	0.25				
	Block (P)*	65.31	7.16	142.2				
	No Phylogeny*					603.35		
	Phylogeny*	64.49	9.96	154.6		569.50	33.7	< 0.001
Flavonoids	Intercept (no P)	12.34	9.96	14.43	< 0.001			
	Intercept (P)	12.36	10.28	14.35	< 0.001			
	Biogeography (no P)	−0.14	−1.49	1.30	0.85			
	Biogeography (P)	−0.05	−1.38	1.35	0.93			
	Height (no P)	−0.03	−0.07	0.02	0.25			
	Height (P)	−0.03	−0.07	0.01	0.20			
	Block (no P)*	0.03	0	0.18				
	Block (P)*	0.02	0	0.03				
	No Phylogeny*					553.54		
	Phylogeny*	0	0	0		553.45	0.22	1
(b) Inducibility of defences								
Condensed tannins	Intercept (no P)	−0.03	−0.001	0.04	0.38			
	Intercept (P)	−723.55	−2798.39	1166.67	0.43			
	Biogeography (no P)	0.74	−0.03	0.04	0.67			
	Biogeography (P)	1090.54	−639.30	2769.50	0.18			
	Height (no P)	−0.01	−0.92	0.81	0.99			
	Height (P)	−0.74	−1.89	0.28	0.19			
	Damage (no P)	−2.20	−0.73	0.76	0.91			
	Damage (P)	−47.83	−129.87	41.62	0.31			
	Block (no P)*	483942	46860	1459575				
	Block (P)*	477719	47624	1394171				
	No Phylogeny*					10524.42		
	Phylogeny*	2053178	443254	4525587		10481.17	43.35	< 0.001

(continued on next page)

Table 2 (continued)

Variable	Source	Posterior mean	1-95% ci ^a	u-95% ci	pMCMC ^b	DIC	ΔDIC	pLLR-test (phylogeny effect)
Hydrolysable tannins	Intercept (no P)	-1.16×10^4	-3.36×10^4	8.99×10^3	0.27			
	Intercept (P)	-5.6×10^3	-1.06×10^5	7.78×10^4	0.91			
	Biogeography (no P)	7.06×10^3	-3.87×10^3	1.84×10^4	0.006			
	Biogeography (P)	8.31×10^3	-7.14×10^4	9.27×10^4	0.82			
	Height (no P)	25.99	-0.78	53.11	0.07			
	Height (P)	2.54	-31.93	39.09	0.89			
	Damage (no P)	-5.03×10^3	-7.84×10^3	-2.80×10^3	< 0.001			
	Damage (P)	-3.58×10^3	-6.44×10^3	-5.63×10^3	0.89			
	Block (no P)*	2.29×10^9	0	7.04×10^9				
	Block (P)*	9.5×10^8	0	3.8×10^9				
	No Phylogeny*					14595.00		
	Phylogeny*	7.9×10^{10}	2.24×10^9	1.47×10^{10}		14509.56	59.83	< 0.001
	Lignins	Intercept (no P)	-943.33	-2499.93	535.58	0.21		
Intercept (P)		-643.58	-6602.28	4591.56	0.80			
Biogeography (no P)		440.52	-310.65	1135.60	0.25			
Biogeography (P)		1316.53	-3272.86	6287.12	0.55			
Height (no P)		0.95	-0.86	2.80	0.32			
Height (P)		-0.88	-3.09	1.67	0.50			
Damage (no P)		-117.49	-263.59	45.48	0.14			
Damage (P)		-196.28	-382.42	13.28	0.05			
Block (no P)*		1360228	60966	3703717				
Block (P)*		1491119	58899	4563193				
No Phylogeny*						11394.42		
Phylogeny*		18752316	3429964	40467003		11349.91	44.44	< 0.001
Flavonoids		Intercept (no P)	302.83	-718.65	1429.35	0.60		
	Intercept (P)	313.51	-811.983	1348.71	0.59			
	Biogeography (no P)	-387.47	-1075.23	359.47	0.28			
	Biogeography (P)	-396.84	-1178.42	217.54	0.27			
	Height (no P)	-0.82	-2.55	0.95	0.37			
	Height (P)	-0.81	-2.51	1.01	0.37			
	Damage (no P)	-146.01	-296.31	3.77	0.06			
	Damage (P)	-147.15	-309.51	-2.01	0.05			
	Block (no P)*	0	0	0				
	Block (P)*	24281	0	127634				
	No Phylogeny*					11362.70		
	Phylogeny*	0	0	0		11362.60	0.16	1
	(c) Leaf herbivory							
Herbivore damage	Intercept (no P)	4.73	3.65	5.69	< 0.001			
	Intercept (P)	3.15	-2.56	8.41	0.24			
	Biogeography (no P)	-1.56	-2.49	-0.66	< 0.001			
	Biogeography (P)	-0.57	-5.21	3.935	0.81			
	Height (no P)	-0.002	-0.005	-0.001	0.03			
	Height (P)	-0.001	-0.003	0.001	0.32			
	Block (no P)*	0	0	0				
	Block (P)*	0	0	0				
	No Phylogeny*					487.38		
	Phylogeny*	17.43	2.39	36.85		449.28	38.71	< 0.001

^a ci = 95% confidence intervals.

^b pMCMC = posterior probability values.

provide a more complete understanding of phylogenetic patterns in constitutive and induced plant defences in *Quercus*.

3.2. Biogeographical differences in oak chemical defence levels and herbivory

Results showed that Nearctic and Palaearctic oak species markedly differed in their constitutive levels of leaf chemical defences. Palaearctic species had significantly higher constitutive levels of condensed tannins than Nearctic ones, whereas the latter group of species had significantly higher constitutive levels of lignins and hydrolysable tannins than Palaearctic species. Higher constitutive levels of condensed tannins have been previously associated with increased herbivore resistance in several species of oaks (e.g., *Quercus robur*; Moreira et al., 2018; Roslin and Salminen, 2008, *Q. ilex*; Solla et al., 2016, *Q. alba* and *Q. velutina*; Forkner et al., 2004) whereas few other studies have reported a positive association between constitutive levels of lignins and herbivory (e.g., *Q. robur*; Moreira et al., 2018, *Q. variabilis*; Wang et al., 2016). This suggests that condensed tannins are a good predictor of herbivore resistance and our results appear to support this affirmation. Indeed, leaf

herbivory was, on average, lower for Palaearctic oak species and we found a negative (marginally significant) association between herbivory and constitutive condensed tannins suggesting these compounds confer resistance. In contrast, lignins and hydrolysable tannins were instead positively correlated with damage suggesting they do not play a defensive role (quite the opposite). These results suggest that Palaearctic species have greater herbivore resistance relative to Nearctic species presumably due to the evolution of higher constitutive levels of condensed tannins (or some other unmeasured trait). We do note, however, that lower herbivory in Palaearctic species could also result from a longer evolutionary history of the interaction between oaks and gypsy moth in Europe, which would have presumably selected for greater resistance to this insect in particular relative to Nearctic species. Thus, generalizations from this pattern should be made with caution, pending findings from further bioassays using other phytophagous insects. In addition, further work addressing the identity and role of individual types of condensed tannins (e.g., Roslin and Salminen, 2008; Pearse and Hipp, 2012) would be relevant to better understand herbivore resistance.

Differences in levels of leaf phenolics for plant taxa of Nearctic vs.

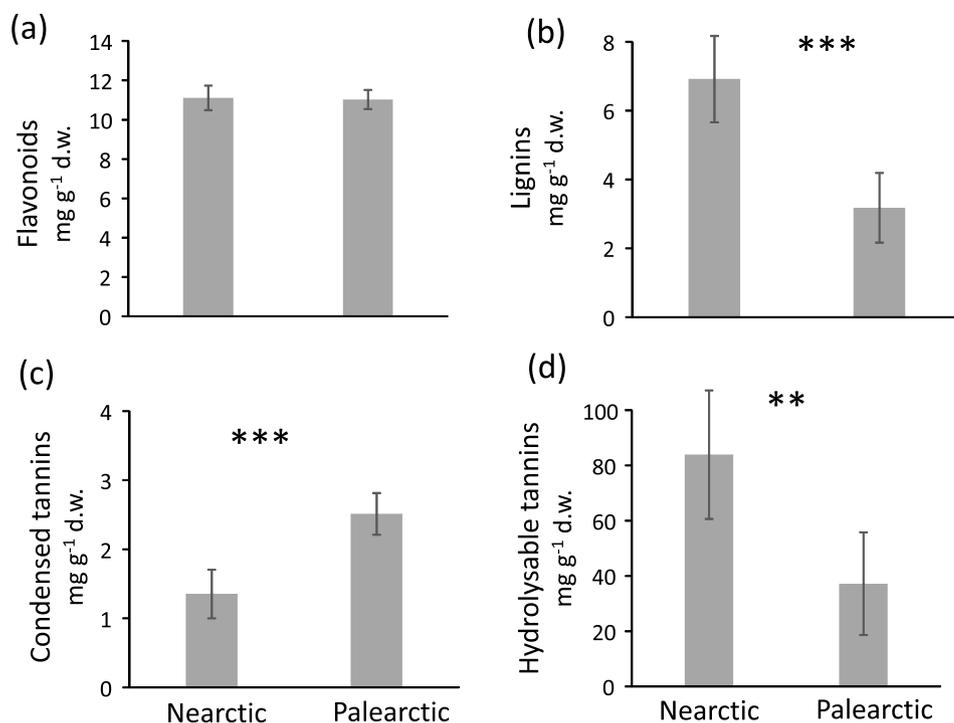


Fig. 2. Constitutive concentration (mg g⁻¹ d.w.) of (a) flavonoids, (b) lignins, (c) condensed tannins and (d) hydrolysable tannins in leaves of one-year-old trees from 11 Palearctic and seven Nearctic *Quercus* species. Bars are least square means \pm standard error (N = 6 replicate plants per *Quercus* species). Asterisks indicate significant differences (*** = $P < 0.001$; ** = $P < 0.01$) among biogeographical regions. Statistics are shown in Table 2a. d.w. = dry weight.

Palearctic regions, particularly condensed tannins, have been reported by other authors (e.g., *Betula* spp.; Muilenburg et al., 2011, *Pinus* spp.; Carrillo-Gavilán et al., 2015, *Quercus* spp.; Pearse and Hipp, 2012), but it is not yet entirely clear which selective agents or historical processes are responsible for these patterns. Proposed explanations include differences between regions in the orientation of geographical barriers, in the duration of biotic interactions between hosts and antagonists along

evolutionary time scales, and in long-term patterns of climatic variability (Archetti et al., 2009; Lev-Yadun and Holopainen, 2009). In the case of deciduous tree species, differences in defence levels have also been attributed to continental-scale differences in herbivore pressure (Archetti et al., 2009; Lev-Yadun and Holopainen, 2009). It has been hypothesized that lower extinction rates of herbivore species during the last glacial period favored greater levels of herbivory (in turn selection

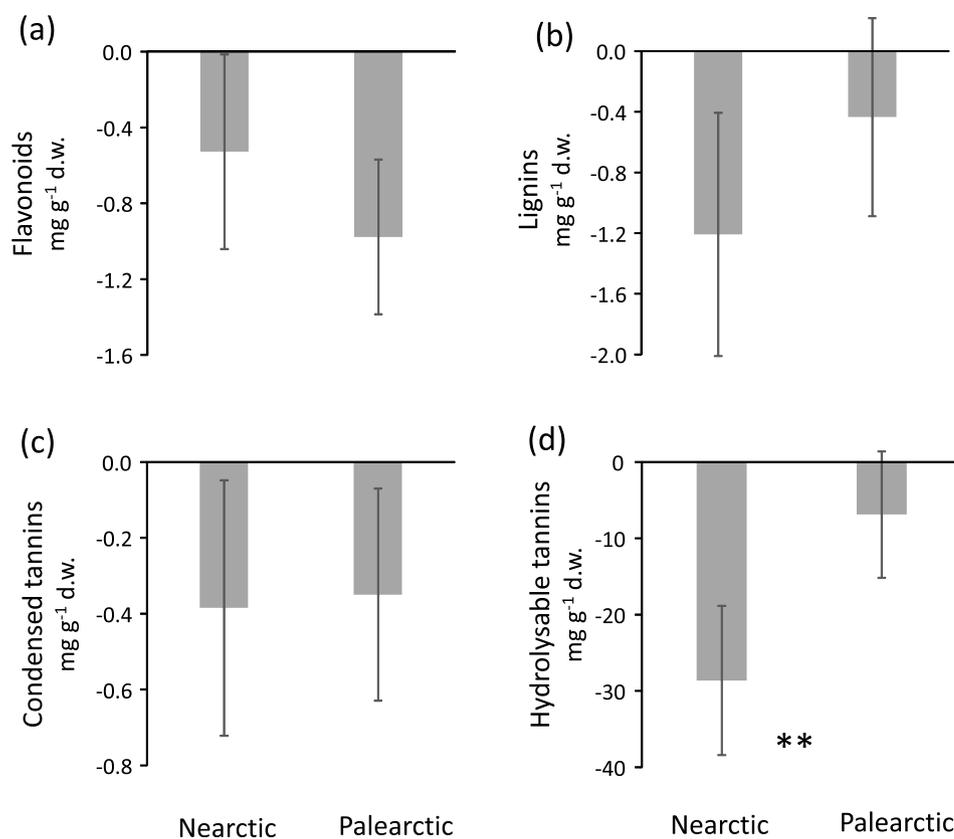


Fig. 3. Inducibility (mg g⁻¹ d.w.) by gypsy moth *Lymantria dispar* larvae of (a) flavonoids, (b) lignins, (c) condensed tannins and (d) hydrolysable tannins in leaves of one-year-old trees from 11 Palearctic and seven Nearctic *Quercus* species. Bars are least square means \pm standard error (N = 6 replicate plants per *Quercus* species). Asterisks indicate significant differences (** = $P < 0.01$) among biogeographical regions. Statistics are shown in Table 2b. d.w. = dry weight.

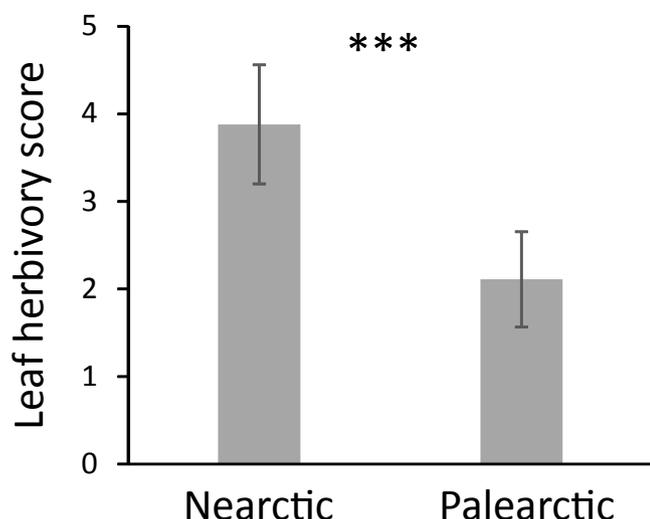


Fig. 4. Leaf herbivory score in one-year-old trees from 11 Palearctic and seven Nearctic *Quercus* species in the greenhouse experiment. Bars are least square means \pm standard error ($N = 6$ replicate plants per *Quercus* species). Asterisks indicate significant differences (***) = $P < 0.001$) among biogeographical regions. Statistics are shown in Table 2c.

for higher defences) in Nearctic deciduous tree species relative to their Palearctic counterpart. Counter to this prediction, we found that the studied Palearctic oaks exhibited, on average, higher levels of condensed tannins – presumably the best predictor of resistance among the compound groups studied – and lower herbivory, suggesting another (as yet unknown) mechanism driving this pattern.

Despite finding no phylogenetic signal for most of the studied traits, accounting for the effect of evolutionary history in the GLMMs in several cases significantly improved model fit, specifically for constitutive levels of lignins and hydrolysable tannins. This suggests that despite weak evidence for phylogenetic inertia in these traits, geographic structuring of phylogenetic patterns was strong enough to account for differences between regions (i.e., differences in defensive phylogenetic constraints across regions). In contrast, for condensed tannins we found a significant phylogenetic signal but evolutionary history apparently did not mediate differences between regions in defence investment, suggesting that observed phylogenetic conservatism in this trait was not geographically structured. Together, these findings highlight the importance of accounting for evolutionary history in conducting broad-scale geographic comparisons of plant defensive traits, as phylogenetic patterns may differ among regions and potentially influence geographic patterns.

In most cases, we found no detectable differences between Nearctic and Palearctic oak species with respect to inducibility of leaf chemical defences (except for hydrolysable tannins). Two previous studies by our group similarly reported that Nearctic and Palearctic pine species did not differ in the inducibility of either resins or phenolics in response to exogenous application of chemical elicitors (jasmonic and salicylic acid) (Carrillo-Gavilán et al., 2015), and due to herbivory by two phytophagous insects (Carrillo-Gavilán et al., 2012). Surprisingly, we found no induction of phenolic compounds after herbivory damage (Fig. SM2 in the Supplementary Material). In fact, most species exhibited induced susceptibility in response to damage, a less common phenomenon which has nonetheless been previously reported for other plant taxa (e.g., Kielkiewicz, 1988; Underwood, 1998). Induced susceptibility may be explained by detoxification of secondary metabolites by gypsy moth larvae; such detoxification would allow increased feeding, and to this the plant may respond by shutting down induced defences, reducing allocation to defences and prioritizing growth (Poreddy et al., 2015). Alternatively, larvae might suppress plant defence

signalling pathways via chemical effectors in their saliva (Poreddy et al., 2015). Further work is necessary to appropriately test these mechanisms. It is also important to note that there was a tendency for greater variability in inducibility than in constitutive defences (compare error bars in Fig. 2 vs. Fig. 3). Aside from intrinsic variability in induced responses, this could have also been due to low leaf sample size and/or variability in herbivore damage driving concomitant variation in inducibility. As a result, this could have precluded the detection of significant effects on inducibility for some of the studied phenolic groups. Further insight would be gained by conducting follow-up work involving greater sampling effort as well as measurements of inducibility of other types of chemical defences (e.g., terpenes) as well as of physical defences (e.g., toughness, trichomes).

4. Conclusions

Our results indicate contrasting patterns of allocation to chemical defences and herbivore resistance between the studied Nearctic and Palearctic *Quercus* species, and these differences might be due to contrasting historical processes or ecological factors acting in each region. In addition, our findings also suggest that phylogenetic inertia in the studied traits influences geographic patterns in defence investment, arguing in favour of accounting for evolutionary history when conducting biogeographical comparisons. Addressing which processes have been the most important contributors to this geographical pattern and the extent to which such effects have shaped the evolutionary history of this genus will require using a greater number of oak species and gathering information on current and historical biotic (e.g., herbivory) and abiotic (e.g., climatic events, orography) conditions.

5. Experimental

5.1. Natural history

To address macro-evolutionary patterns of plant defences allocation, we used 18 oak species, of which 11 have a Palearctic distribution range, namely: *Quercus robur*, *Q. faginea*, *Q. petraea*, *Q. suber*, *Q. ilex*, *Q. pubescens*, *Q. frainetto*, *Q. phillyraeoides*, *Q. cerris*, *Q. serrata* and *Q. pyrenaica*, and seven have a Nearctic distribution, namely: *Q. agrifolia*, *Q. macrocarpa*, *Q. coccinea*, *Q. rubra*, *Q. shumardii*, *Q. palustris* and *Q. phellos* (Manos et al., 1999; Hipp et al., 2018) (Fig. 1).

Quercus species support a large community of specialist and generalist insect herbivores, mainly leaf chewers and miners (e.g., Roslin and Salminen, 2008; Pearse and Hipp, 2009; Tack and Roslin, 2011; Moreira et al., 2017, 2018). Among these herbivores, the generalist gypsy moth (*Lymantria dispar*, Lepidoptera) is one of the most destructive pests of broad-leaf and conifer trees in Western Europe (Milanovic et al., 2014). This insect is an increasingly important ecological and economic pest in North America where it was accidentally introduced in 1868 (Elkinton and Liebhold, 1990). Larvae of this species feed at night on leaves where they chew small holes and cause extensive damage on large amounts of leaves (Miller and Hanson, 1989). Although co-evolution of oaks and the gypsy moth might have mainly occurred in the Palearctic region, this phytophagous insect could be viewed as a good candidate to test inducibility of quantitative defences (i.e., the ability to increase constitutive levels in response to damage) and resistance against herbivores because leaf chemistry is relatively similar between oak species from different regions and gypsy moth is a generalist that feeds on many species of Nearctic *Quercus* (Elkinton and Liebhold, 1990).

Quercus species produce chemical defences in leaves, particularly phenolic compounds, which are effective against a broad range of phytophagous insects (Feeny, 1970; Roslin and Salminen, 2008; Pearse and Hipp, 2009; Abdala-Roberts et al., 2016; Moreira et al., 2018). Constitutive levels of phenolic compounds are present at high concentrations in all plant tissues (up to 100 mg g⁻¹ tissue dried weight)

(Pearse and Hipp, 2012; Moreira et al., 2017, 2018). Additionally, inducibility of these chemical defences has been shown to provide an effective strategy for resistance against major insect herbivores in several *Quercus* species (e.g., Mizumachi et al., 2012).

5.2. Experimental design

We conducted a greenhouse experiment using 12 plants of each oak species. We added gypsy moth larvae to half of the plants of each oak species, whereas the other half were not damaged (controls). The experiment followed a randomized split-plot design replicated over six blocks, where herbivore induction was applied at the whole-plot factor and oak species was the split factor. This design comprised six blocks \times 18 oak species \times two induction treatments (control and induction by gypsy moth larvae), for a total of 216 plants.

5.3. Experimental conditions, trait measurements and sampling

In March 2017, we individually planted one-year-old plants (purchased from Planfor nursery, Uchacq-et-Parentis, France) in 4-L containing potting soil with peat in a glass greenhouse. Plants were grown under controlled light (minimum 12 h per day, Photosynthetically Active Radiation = $188 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$) and temperature (10 °C night, 25 °C day), and were watered daily. Two months after planting, we measured total height of each plant (“plant initial height” hereafter) and randomly assigned half of the plants of each species to one of two treatments: (1) herbivore-induced plants with gypsy moth larvae and (2) undamaged control plants. For each plant assigned to the induction treatment, we placed a fourth-instar larvae on fully expanded leaves. Larvae were allowed feeding on plants for four days, after which, we visually estimated percent leaf area removed at the whole-plant level by using the following scale: 0 = undamaged; 1 = 1–10% damaged; 2 = 11–20% damaged; 3 = 21–30% damaged; 4 = 31–40% damaged; 5 = 41–50% damaged; 6 = 51–60% damaged; 7 = 61–70% damaged; 8 = 71–80% damaged; 9 = 81–90% damaged; 10 = 91–100% damaged. To avoid biases in our herbivory estimates, the same person (XM) scored all the leaves. After assessing levels of damage, we immediately collected three randomly chosen leaves from each plant for subsequent quantification of phenolic compounds, as well as two more leaves from three constitutive plants of each species for molecular analyses to build the phylogenetic tree of *Quercus* species. Leaves for phenolic analyses were oven-dried for 48 h at 40 °C, ground with liquid nitrogen, and stored at room temperature. To prevent DNA degradation, leaves for molecular analyses were immediately stored in the freezer at -80 °C.

5.4. Chemical analyses

We extracted phenolic compounds using 20 mg of dry plant tissue with 1 mL of 70% methanol in an ultrasonic bath for 15 min, followed by centrifugation (Moreira et al., 2014). We diluted these methanolic extracts with 70% methanol (1:5 vol:vol) and transferred them to chromatographic vials to perform the chromatographic analyses. We carried out the chromatographic analyses on an Ultra-High-Performance Liquid-Chromatograph (UHPLC Nexera LC-30AD; Shimadzu) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector. The UHPLC column was a Kinetex™ 2.6 μm C18 82–102 Å, LC Column 100 \times 4.6 mm, protected with a C18 guard cartridge. The flow rate was 0.4 mL min^{-1} and the oven temperature was set at 25 °C. The mobile phase consisted of two solvents: water-formic acid (0.05%) (A) and acetonitrile-formic acid (0.05%) (B), starting with 5% B and using a gradient to obtain 30% B at 4 min, 60% B at 10 min, 80% B at 13 min and 100% B at 15 min. The injection volume was 30 μL . We processed data on a computer with the LabSolutions software (Shimadzu). We quantified flavonoids as rutin equivalents, condensed tannins as catechin equivalents, hydrolysable

tannins as gallic acid equivalents, and lignins as ferulic acid equivalents (Moreira et al., 2018). We achieved the quantification of these phenolic compounds by external calibration using calibration curves at 0.25, 0.5, 1, 2 and 5 $\mu\text{g mL}^{-1}$ (Moreira et al., 2018). We expressed phenolic compound concentrations in mg g^{-1} tissue on a dry weight basis.

5.5. Molecular analyses

We built a phylogenetic tree of *Quercus* species based on Single Nucleotide Polymorphism (SNP) matrices by ddRAD sequencing (Fig. 1). For this, we extracted DNA following Pandey and Tamta (2015), purified it using the Isolate II Plant DNA kit (Bioline, London, UK) and quantified it by fluorimetric methods (Qubit, Thermo Fisher Scientific).

We prepared low-representation libraries from each DNA sample using an in-house developed protocol modified from Kess et al. (2016). We digested DNA with *Bgl*II and *Pst*I and attached adapters specific to the digested fragments with a T4 DNA Ligase (NEB). In order to enrich the libraries with adapter-ligated fragments the libraries were subjected to a PCR step with a Phusion High-Fidelity DNA Polymerase (NEB). Then, we attached dual-index combinations to each end of the adapter-ligated fragments to allow for multiplexing different libraries in the same HiSeq 2500 PE100 platform (Illumina) lane. After this, we purified libraries using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek), quantified them by fluorimetric methods (Qubit, Thermo Fisher Scientific), pooled them in equimolar amounts and finally we retrieved a band of approximately 700 base pairs from an agarose gel.

We quality-checked raw fastq files in FastQC 0.11.3. Then, we used Trimmomatic 0.36 to (i) remove adapters (ILLUMINACLIP option), (ii) check enzyme cut sites and (iii) fix the same length for all the reads employing Bolger et al. (2014). We used BWA 0.7.12 to map the reads to the *Q. lobata* assembly (accession number: GCA 001633185) (Li and Durbin, 2010). We used SAMtools 0.1.19 to remove low quality and secondary alignments (Li et al., 2009), Qualimap 2.2.1 to observe the coverage distribution (Okonechnikov et al., 2015) and the STACKS 1.44 software to perform the genotyping of the reads and also in the pre-processing step (Catchen et al., 2013). Ustack parameters were settled as $m = 10$, $N = 0$ and $M = 3$. We used the Populations program included in the STACKS pipeline to generate the final data matrices.

We used RAxML v8 software for the phylogenetic reconstruction of the *Quercus* phylogeny based on SNP matrices that included variable sites. We enabled the ascertainment bias correction for the GTRGAMMA model ($-m$ ASC GTRGAMMA) and we tested the two types of corrections ($-\text{err-corr} = \text{lewis}$ and $-\text{asc-corr} = \text{felsenstein}$) described in Leaché et al. (2015). We activated the RAxML option for rapid bootstrapping and the number of bootstraps were automatically determined using the option $\#- \text{autoMRE}$.

5.6. Statistical analyses

We first estimated the phylogenetic signal (lambda value, λ) for constitutive and induced levels of each group of phenolic compounds as well as for leaf herbivory using species-level averages with the function *phylosig* of the package *phytools* in R (Revell, 2012). A lambda value of 1 indicates phylogenetic conservatism consistent with the tree topology and a random walk model (i.e., herbivory or defence similarity is directly proportional to the extent of shared evolutionary history). A lambda value of 0 indicates no influence of shared ancestry on herbivory or defence (i.e., phylogenetic independence).

We then proceeded to test for the effect of biogeographical region (Palearctic vs. Nearctic) and evolutionary history (phylogenetic relationships) on constitutive levels and inducibility of each group of chemical defences with Generalized Linear Mixed Models using Markov chain Monte Carlo techniques (MCMC GLMMs) (Package MCMCglmm in R; Hadfield, 2010). Although our analysis included more Palearctic species ($N = 11$) than Nearctic ones ($N = 7$), GLMMs are considerably

robust to unbalanced designs (Littell et al., 2006). In the case of constitutive levels, we ran models using data at the plant level separately for each group of phenolic compounds using only control (undamaged) plants ($N = 216$). For each group of chemical defences we first ran a full model including biogeographical region as a fixed factor, initial plant height as covariate (because growth may influence plant defence production), as well as block as a random factor. We then ran again the model but including also the phylogenetic variance-covariance matrix as a random factor to assess the influence of evolutionary history on biogeographical differences in defence production. Assuming Brownian motion, the expected covariance is directly proportional to the amount of shared evolutionary history. The diagonal elements of the matrix (variance) represent the total branch length from root to tip for each species, while the off-diagonal elements represent the branch length shared by respective species pairs. For each defensive trait, we compared models with and without this factor using the Deviation Information Criterion (DIC), where a significantly smaller DIC value for the model with this factor ($\Delta DIC > 5$ without vs. with evolutionary history in the model) (Spiegelhalter et al., 2014) indicates that evolutionary history significantly influences defences. Accordingly, if significant differences between biogeographical regions in defence levels are shaped by shared evolutionary history of the studied taxa (i.e., geographically structured phylogenetic pattern), then a significant effect of biogeographical region in the first model should turn non-significant after accounting for this factor. In the case of inducibility of defences, constitutive and induced levels for a given group of compounds cannot be determined simultaneously for a single plant because collecting tissue to estimate constitutive levels necessarily influences subsequent measurements of induced levels. We therefore used a bootstrapped approximation on our data as the best approach for replicating within-species variation in inducibility following Moreira et al. (2013). We estimated inducibility of phenolic compounds in each plant as the difference between the concentration of each induced plant and that of six control plants of the same species (concentrations of control plants represent constitutive levels). This resulted in six estimates of phenolic inducibility which were treated as repeated measures on the same subject (Moreira et al., 2013). As for constitutive defences, we first ran MCMC GLMMs testing for effects of biogeographical region on inducibility separately for each group of phenolic compounds using plant-level data. Subsequently, we ran again each model but also including evolutionary history (estimated as explained above for constitutive levels) as a random factor and compared model DIC values as well as the significance of the effect of biogeographical region in the models without vs. with this factor. In both sets of inducibility models, we also included plant initial height and percentage of leaf area removed by gypsy moth as covariates, as well as block as a random factor. Finally, we also tested for inter-specific variation in constitutive defences and their inducibility for each group of compounds with mixed models including oak species (fixed factor) and block (random factor) as predictors of each defensive trait.

As for chemical defences, leaf herbivory was also modelled using MCMC GLMMs as described above, first evaluating the effect of biogeographical region and then adding the effect of evolutionary history to determine whether this factor is an important predictor of leaf damage, as well as if it mediates differences in the amount of herbivory between oak species from each region. In addition, we also tested inter-specific variation in the amount of leaf damage using a mixed model that included the effects of oak species (fixed factor) and block (random factor). To assess whether patterns of damage were associated with variation in defence levels, we performed species-level correlations between leaf herbivory and the constitutive concentration of each group of compounds (flavonoids, lignins, condensed tannins, and hydrolysable tannins). These tests accounted for phylogenetic non-independence based on maximum likelihood phylogenetic generalized least-squares (Pagel, 1999).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.phytochem.2018.06.002>.

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