



ORIGINAL
ARTICLE



Phylogenetic and biogeographical patterns in defensive strategies and quantitative allocation to chemical defences in Palaeartic and Nearctic pine trees

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ABSTRACT

Aim Using pine trees as a long-lived woody plant model, we analysed the extent to which constitutive and induced allocation to chemical defences were conserved across the phylogeny, and tested the hypothesis that patterns of defensive allocation and defensive strategies differ between Palaeartic and Nearctic pines.

Location Common-environment study with Palaeartic and Nearctic pine species.

Methods We grew 17 pine species (eight Palaeartic and nine Nearctic) in a common environment and measured the stem and needle concentrations of phenolics and non-volatile resin, two major quantitative chemical defences in pines. In particular, we studied the constitutive allocation to chemical defences (in control plants) and the potential inducibility of those traits in response to elicitation with hormonal analogues of the jasmonic acid (methyl jasmonate) and salicylic acid (benzothiadiazole) pathways of plant defence against herbivorous insects and pathogens.

Results We found a strong phylogenetic signal in the constitutive concentration of phenolics in the needles and resin in the stem, but not elsewhere. We found that Nearctic pines showed 70% greater concentration of constitutive needle phenolics than Palaeartic species. In contrast, the inducibility potential of defences did not differ between Nearctic and Palaeartic pine species.

Main conclusions Our findings suggest that macroevolutionary history (phylogeny and biogeographical origin combined) has played an important role in the evolution of needle constitutive defences among pine species, but not in that of induced defences. Specifically, constitutive allocation to pine chemical defences seems to be well preserved across the phylogeny. In contrast, the inducibility of chemical defensive traits was evolutionarily labile and may depend more on the particular climate, biotic interactions and resource availability in each species' range.

Keywords

Constitutive defences, induced defences, inducibility, oleoresin, phenolics, *Pinus*, plant–herbivore interactions.

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INTRODUCTION

Understanding the determinants of phenotypic variation in antiherbivore plant defences across species has been a major research focus for over three decades (Coley *et al.*, 1985; Stamp, 2003; Futuyama & Agrawal, 2009; Karban, 2011). A

number of studies have proposed that biotic (e.g. herbivory) and abiotic (e.g. climate and resource availability) factors are the fundamental drivers explaining the allocation to, and ultimately the evolution of, plant defences (Rhoades & Cates, 1976; Coley *et al.*, 1985; Stamp, 2003). Classic and recent investigations have suggested that the evolutionary relationships

among plants, as well as between plants and herbivores, could also explain the distribution of defences among plant species (Ehrlich & Raven, 1964; Agrawal *et al.*, 2009a; Pearse & Hipp, 2009; Desurmont *et al.*, 2011). Both phylogeny and biogeography are drivers of herbivore abundance and diversity and consequently key predictors of plant defensive strategies. Because closely related plant species generally host similar herbivores (Ehrlich & Raven, 1964; Weiblen *et al.*, 2006; Futuyama & Agrawal, 2009; Pearse & Hipp, 2009; Craft *et al.*, 2013), they are also likely to share similar defensive chemistry (Garland *et al.*, 1992; Wink, 2003; Agrawal *et al.*, 2009a; Armbruster *et al.*, 2009). For instance, Agrawal *et al.* (2009b) observed substantial phylogenetic signal for some chemical (cardenolides and latex) and mechanical (trichomes) defensive traits in 53 species of *Asclepias* (Apocynaceae), indicating that much of their variation was predicted by the evolutionary relationships among species. Furthermore, biogeographical factors, even at the continental scale, might strongly constrain the evolution of plant antiherbivore defences by determining the array of biotic interactions in both terrestrial and marine communities (Bryant *et al.*, 1994; Steinberg *et al.*, 1995; Jenkins *et al.*, 2008; Desurmont *et al.*, 2011; Craft *et al.*, 2013). During the last ice age, differences in the orientation of the main geographical barriers, and long-term climate variability between the Nearctic (i.e. North America) and the Palaearctic (i.e. northern Eurasia), have produced marked differences in extinction rates, demographic history and recolonization both of plants and their herbivores. The duration of biotic interactions between hosts and antagonists along evolutionary time also differs between regions, and these factors have together conditioned the evolution of plant defensive traits within each region (Archetti *et al.*, 2009; Lev-Yadun & Holopainen, 2009). For instance, Desurmont *et al.* (2011) reported that shared history with common herbivores determined resistance across 16 *Viburnum* (Adoxaceae) species in North America and Eurasia. Conspicuous differences in red and brown autumn coloration of the leaves of deciduous trees between Nearctic and Palaearctic continental ranges have also been suggested to reflect differing herbivore pressures in the evolutionary past (Archetti *et al.*, 2009).

Although phylogenetic and biogeographical patterns can be major drivers of variation in plant antiherbivore resistance, not all data have supported this view, with some studies reporting weak phylogenetic signals for plant resistance traits (e.g. Kursar *et al.*, 2009; Kempel *et al.*, 2011; Rasmann & Agrawal, 2011a). One possible factor underlying this lack of consistency might be that few studies have tested the influence of phylogeny and biogeographical patterns on plant defensive strategies by separating constitutive investment (which is always expressed) and induced investment (produced in response to herbivore damage or pathogen infection) in chemical defences (but see Thaler & Karban, 1997; Kempel *et al.*, 2011; Rasmann & Agrawal, 2011a). In addition to this, little is known about the patterns of variation in defensive strategies across species of long-lived woody plants (e.g. trees) with life-history determinants and evolutionary patterns that

greatly differ from those of annual and herbaceous plants (Petit & Hampe, 2006). The relative costs and benefits of constitutive and induced resistance depend on resource availability and on herbivore pressure, predictability and feeding behaviour (Agrawal, 2007; Sampedro *et al.*, 2011), and these factors could contribute to the differing biogeographical patterns of defensive strategies across tree species.

In this study, we examine general patterns in the quantitative allocation to constitutive and induced chemical defences in pine species. Pines are a good model in which to study phylogenetic and biogeographical patterns of plant defences, because they occupy broad edaphic and climatic ranges (Richardson, 1998). Defensive chemistry in pine trees, as in most conifers, is based on very high concentrations of carbon-based compounds in their tissues, also known as 'quantitative defences', mainly terpenoid oleoresin and phenolics (Franceschi *et al.*, 2005; Mumm & Hilker, 2006). Pines have been subjected to selection from different abiotic and biotic conditions, which has led to marked differences in chemical defences and defensive strategies among species (Zas *et al.*, 2011; Carrillo-Gavilán *et al.*, 2012). We previously studied the role of climate as a determinant of pine defensive patterns (Moreira *et al.*, 2014), and here we focus specifically on whether phylogeny and biogeography also contribute to the patterns of defensive strategies in pine trees.

We analysed the constitutive concentration of non-volatile resin and total phenolics in two tissues with contrasting fitness value and herbivore pressure (needles and stem) in saplings of 17 pine species of Palaearctic or Nearctic origin, grown in a common greenhouse environment. We also assessed the inducibility of these chemical traits (the ability to increase constitutive levels in response to damage) because inducibility represents a major determinant of resistance to insects and early-stage survival in conifers (Zhao *et al.*, 2011; Schiebe *et al.*, 2012). We evaluated the potential inducibility associated with damage-signalling by the two main defensive hormonal pathways, jasmonic acid (JA) and salicylic acid (SA). The JA pathway is activated in response to chewing insects and necrotrophic pathogens, whereas the SA pathway is involved in plant responses to phloem-suckers and biotrophic pathogens (Pieterse *et al.*, 2012). Specifically, we tested (1) whether constitutive and induced strategies differ in their phylogenetic conservatism, and (2) whether patterns of pine chemical defensive investment and defensive strategies differ between continents. Overall, by addressing both questions, our work constitutes a first step towards understanding the phylogenetic and biogeographical patterns in defensive strategies in some of the Northern Hemisphere's most ecologically and economically important trees.

MATERIALS AND METHODS

Study species and experimental design

Seventeen pine species (Pinaceae: *Pinus*), all of which occupy large natural or planted areas worldwide, were used for this

study (see Table S1 in Appendix S1 of the Supporting Information). *Pinus canariensis*, *P. halepensis*, *P. nigra* subsp. *salzmannii*, *P. nigra* subsp. *laricio*, *P. pinea*, *P. pinaster*, *P. sylvestris* and *P. roxburghii* have Palaeartic ranges, whereas *P. banksiana*, *P. contorta*, *P. coulteri*, *P. muricata*, *P. patula*, *P. ponderosa*, *P. radiata*, *P. sabiniana* and *P. taeda* have Nearctic ranges (Richardson, 1998). Arising in the Mesozoic, the subgenus *Pinus* is divided into two distinct lineages, corresponding to Eurasian and North American ranges (Millar, 1998; Price *et al.*, 1998). The former was further differentiated into two clades, the subsection *Pinaster* (the Mediterranean pines) and the subsection *Pinus* (Geada López *et al.*, 2002; Grivet *et al.*, 2013).

We conducted a factorial greenhouse experiment using the 17 pine species and induction of plant defensive responses as the main factors. Induction included three experimental treatments: control plants for the constitutive level; induction of quantitative chemical defences associated with the JA pathway; and induction of chemical defences associated with the SA pathway. The experiment followed a randomized split-plot design replicated over 10 blocks, with induction of plant defences as the whole-plot factor, and pine species as the split factor. Owing to a lack of plant material, only 15 pine species were used in the SA treatment. In total, we grew 490 pine seedlings, corresponding to 10 blocks × 17 species × 2 treatments (control and induction of the JA pathway), plus 10 blocks × 15 species × 1 treatment (induction of the SA pathway).

Greenhouse conditions and defence induction treatments

In October 2008, pine seeds were individually sown in 2-L pots filled with a mixture of perlite and peat (1:1 v/v), fertilized with 12 g of a slow-release fertilizer (Multicote N:P:K 15:15:15), and covered with a 1–2-cm layer of sterilized sand. To avoid interference from pathogens, seeds were treated with a fungicide before sowing (Fernide, Syngenta Agro, Spain). Pots were placed in a glass greenhouse at the Centro de Investigación Forestal de Lourizán (Xunta de Galicia, Pontevedra, Spain) with controlled light (minimum 12 h per day) and temperature (10 °C night, 25 °C day), and daily watering. For more detailed information, see Carrillo-Gavilán *et al.* (2012).

Ten months after sowing, pine saplings were randomly assigned to one of three treatments: 40 mM methyl jasmonate (MJ; Sigma-Aldrich, Madrid, Spain); 5 mM of benzo-(1,2,3)-thiadiazole-7-carbothioic S-methyl ester (BTH, Bion 50WG; Syngenta Agro, Spain) or treated with the carrying solution (2.5% ethanol in water; control plants). MJ and BTH are synthetic functional analogues that trigger the JA and SA pathways of plant defensive signalling, respectively. Treatments were applied once: plants were sprayed evenly over the foliage with a handheld sprayer until runoff occurred. The dose and concentration of both chemical induction treatments were determined based on previous

studies (Moreira *et al.*, 2009, 2012a,b). To avoid cross-contamination, the two induction treatments were applied in different rooms of the greenhouse, and plants remained there to dry for 48 h.

Plant traits and chemical analyses

Two weeks after application of the chemical induction treatments, when the induction effects were assumed to be greatest (see Martin *et al.*, 2002), plant height was measured and plants were harvested above the root collar. Subsamples of the basal part of the stem (*c.* 5 cm) and the needles (*c.* 2 g) were immediately collected from each individual pine, flash-frozen and stored at –30 °C for chemical analysis.

Concentration of non-volatile resin in the stem and needles was estimated gravimetrically following the procedure described by Moreira *et al.* (2012c). Non-volatile resin was quantitatively extracted twice with *n*-hexane in an ultrasonic bath, after which the plant material was recovered by filtration (Whatman GFF, Maidstone, UK), the solvent in the tubes was evaporated to dryness, and the mass of the non-volatile resin residue was determined with a precision scale to the nearest 0.0001 g. This determination of non-volatile resin was a good proxy of the concentration of diterpenes ($r = 0.921$; $P < 0.001$) as quantified by gas chromatography (Sampedro *et al.*, 2011). Non-volatile resin concentration was expressed in milligrams of non-volatile resin per gram dry weight (d.w.) of plant tissue.

Stem and needle subsamples for the determination of total phenolics were oven-dried at 45 °C to constant weight, manually ground in a mortar with liquid nitrogen and extracted and analysed as described by Moreira *et al.* (2012c). Briefly, phenolics were extracted with aqueous methanol (1:1 v/v) in an ultrasonic bath for 15 min, followed by centrifugation and subsequent dilution of the methanolic extract. Total polyphenolic concentration was estimated colorimetrically by the Folin–Ciocalteu method in a Bio-Rad 650 microplate reader (Bio-Rad Laboratories, Philadelphia, PA, USA) at 740 nm, using tannic acid as standard, and expressed on a dry-weight (d.w.) basis.

Statistical analyses

Preliminary analyses of the effects of block, induction treatment and pine species on the concentration of the two chemical compounds in each tissue were performed using a mixed model in PROC-MIXED, SAS System (Littell *et al.*, 2006; see Appendices S2 & S3). Inducibility was estimated as the difference in concentration of chemical defences between the existing basal constitutive level and that in induced plants. In pine trees, however, the constitutive and induced concentrations of chemical defences could not both be assessed for the same individual, because destructive sampling of the entire plant was required. We therefore used a bootstrapped approximation on our data as the best approach for replicate within-species variation in

inducibility, following Moreira *et al.* (2013a). The inducibility of each plant was estimated as the difference between the induced value for a given plant and the value for the other 10 plants of the same species in the constitutive treatment. This resulted in 10 estimates of inducibility for each induced plant, which were treated as repeated measures on the same subject. Because we found that there were significant effects of induction treatments and that within-species variance was lower than between-species variance (a basic assumption of phylogenetic comparative analyses), we used phylogenetic comparative methods to control for the non-independence of data points due to shared ancestry (Freckleton *et al.*, 2002). We used the phylogenetic tree published by Eckert & Hall (2006) (see Fig. S1 in Appendix S1), with branch lengths estimated as the number of expected substitutions. The phylogenetic tree was trimmed prior to the analyses to include only the species of interest and made ultrametric through nonparametric rate smoothing (Sanderson, 1997) with the package *APE* (Paradis *et al.*, 2004) in R (R Development Core Team, 2008) (Fig. 1). For all analyses, data were log-transformed to fulfil the assumptions of the evolutionary models.

Second, in order to determine whether there was an effect of the distribution range (Palaeartic versus Nearctic) on the constitutive levels and inducibility of chemical defences, we used phylogenetic generalized least-squares models (PGLS; Martins & Hansen, 1997). We entered the chemical defences as a response variable and the distribution range as the main factor. The analyses assumed a Brownian motion model of evolution and a maximum-likelihood estimate of the λ parameter that was estimated simultaneously with model fit (Martins & Hansen, 1997; Pagel, 1999; Freckleton *et al.*,

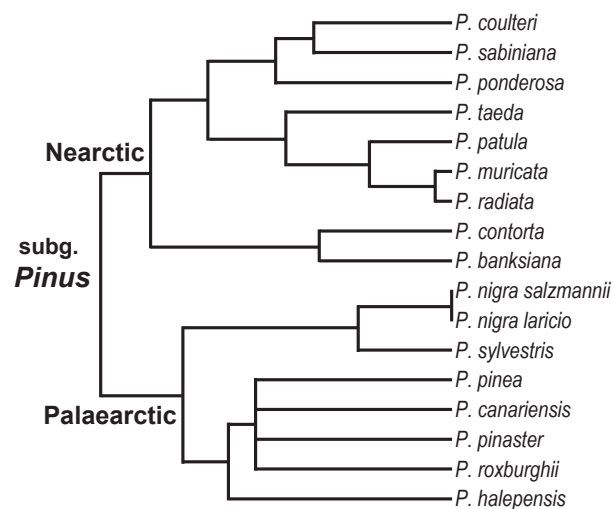


Figure 1 Phylogenetic tree of the studied *Pinus* species according to that by Eckert & Hall (2006), based upon chloroplast sequences at four loci covering 83 pine species. Only subgenus *Pinus* is shown. Biogeographical regions are indicated at the nodes. Note that the apparent four-species polytomy of Palaeartic species actually conceals bifurcating relationships with branch lengths too short to be apparent in the figure.

2002). We carefully verified that the assumptions of each model were met (Freckleton, 2009). Although the *Pinus* phylogeny presents an early split into a Palaeartic and a Nearctic clade, and there is therefore likely to be strong covariation between biogeographical and phylogenetic effects, the use of PGLS methods allows us to control for non-independence due to shared ancestry as well as the distinct evolutionary trajectories between species within each biogeographical area.

RESULTS

Species differed significantly (mixed models, $P < 0.024$) in their concentrations of non-volatile resin and total phenolics in the stem and the needles, both in control plants and after induction with MJ and BTH (Appendix S2). The concentration of non-volatile resin was generally more variable among species than the total concentration of phenolics. The coefficients of variation of the mean (CV) across species were around 10–15% for the concentration of non-volatile resin, and slightly less for total phenolics (CV 5–10%). Within species, CV was about 15–28% for the 12 chemical traits analysed. The minimum CV was for constitutive resin in the stem of *P. nigra* (CV = 5.7%), and the greatest variability in estimation within species was for MJ-induced resin in the needles of *P. patula* (CV 78.7%) (Appendix S2).

There was a strong phylogenetic signal in the constitutive levels of total phenolics in the needles and non-volatile resin in the stem. Estimates of λ were close to 0.85 (Table 1), significantly greater than zero in both cases, and not significantly different from 1 in the case of needle phenolics, indicating that between-species differences in these defensive traits were strongly influenced by the phylogenetic relatedness of species. In contrast, constitutive levels of non-volatile resin in needles and phenolics in stem were not influenced by the phylogenetic relationships between species, with λ values not significantly different from zero (Table 1). The JA-associated inducibility of both defensive traits showed very little phylogenetic signal, not significantly different from zero in both tissues (Table 1). We observed a depletion of non-volatile resin in the stem after application of SA, i.e. an induced susceptibility effect (Appendix S2), which showed a very strong phylogenetic signal, suggesting an evolutionarily conserved pattern of SA-mediated responses in pine trees (Table 1).

When testing our hypothesis that the Nearctic and Palaeartic species could differ in chemical defensive investment and defensive strategies, we found that Nearctic pine species had close to 70% greater constitutive concentration of total phenolics in the needles than their relatives in the Palaeartic region (Fig. 2d). Pine trees from both biogeographical regions did not differ, however, in the constitutive allocation of total phenolics in the stem, or non-volatile resin in the stem and in needles (Fig. 2). Inducibility of total phenolics in needles associated with JA signalling was four times greater in Palaeartic pines than those from the Nearctic,

Table 1 Analysis of the phylogenetic signal (λ ; Pagel, 1999) in the constitutive allocation to chemical defences (non-volatile resin and total phenolics) and their inducibility in 17 Palaeartic and Nearctic pine (*Pinus*) species. The potential inducibility of chemical defences associated with the jasmonic acid (JA) and salicylic acid (SA) pathways are shown. Concentrations of chemical defences were measured in two tissues (stem and needles). The maximum-likelihood estimate of λ , an estimate of the phylogenetic signal, and its associated log-likelihood values ('ln lik') are shown; λ varies between 0 (no phylogenetic signal) and 1 (strong phylogenetic signal or complete phylogenetic dependence). Log-likelihood values of the model when λ was forced to $\lambda = 1$ and $\lambda = 0$ are also shown; cases where the λ value differed significantly from 1 or 0 according to the log-likelihood ratio test (Freckleton *et al.*, 2002) are shown in bold type.

Defensive status and chemical defence	Tissue	Evolutionary model			
		λ	ln lik	ln lik ($\lambda = 1$)	ln lik ($\lambda = 0$)
Constitutive					
Non-volatile resin	Stem	0.84	3.35	0.00*	-0.06*
	Needles	0.00	2.19	-1.31**	2.19 n.s.
Total phenolics	Stem	0.00	11.12	10.07 n.s.	11.12 n.s.
	Needles	0.87	7.38	7.06 n.s.	4.42*
Inducibility by JA					
Non-volatile resin	Stem	0.06	37.57	35.05*	37.56 n.s.
	Needles	0.00	21.23	17.94*	21.23 n.s.
Total phenolics	Stem	0.00	38.59	34.03**	38.59 n.s.
	Needles	0.10	30.96	22.17***	30.92 n.s.
Inducibility by SA					
Non-volatile resin	Stem	1.00	34.36	34.36 n.s.	31.89*
	Needles	0.00	29.30	25.39*	29.30 n.s.
Total phenolics	Stem	0.00	40.02	32.32***	40.02 n.s.
	Needles	0.00	32.62	27.94**	32.62 n.s.

n.s. not significant; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0001$.

and this was marginally significant (Fig. 3d). There were, however, no significant differences across regions in the inducibility of either the other chemical defences elicited by JA (Fig. 3), or those associated with SA signalling (Fig. 4).

DISCUSSION

We found a strong phylogenetic signal in the constitutive concentration of total phenolics in the needles and non-volatile resin in the stem. A strong phylogenetic signal is consistent with the evolutionary conservatism of these traits, and indicates that patterns of variation might be predicted from the evolutionary relationships among pine species. We also found a strong phylogenetic signal in the SA-mediated depletion of non-volatile resin in the stem, which may be related to antagonistic physiological crosstalk between SA-mediated signalling pathways linked to biotic and abiotic stress (Fujita *et al.*, 2006). When the results for inducibility of chemical defences are taken together across tissues and signalling pathways, however, the overall trend indicates that inducibility traits seem to be more evolutionarily labile, showing weak phylogenetic signal. Our results agree with the hypothesis that chemical defences that evolve in response to selection imposed by herbivores allow the plant lineage to radiate into diverse species that share the new effective anti-herbivore defence. This leads to phylogenetic conservatism, whereby more closely related plant species share similar chemical defences (Agrawal, 2007). Some studies reporting strong phylogenetic signal in diverse plant defensive traits have supported an 'escape-and-radiate' model (e.g. Garland *et al.*, 1992; Wink, 2003; Agrawal *et al.*, 2009a,b; Armbruster *et al.*, 2009; Rasmann & Agrawal, 2011b); other studies have found weak or no phylogenetic signal in key herbivore resistance traits (e.g. Kursar *et al.*, 2009; Kempel *et al.*, 2011; Rasmann

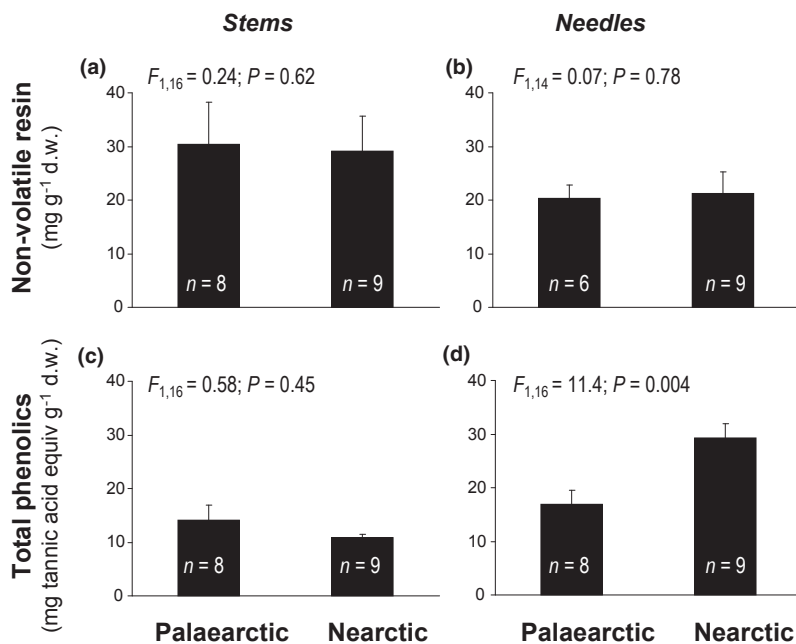


Figure 2 Constitutive concentration of quantitative chemical defences (a–b, non-volatile resin; c–d, total phenolics) in (a,c) the stem and (b,d) needles of Palaeartic and Nearctic pine species. F and P -values of the effect of biogeographical region of distribution after controlling for phylogeny using PGLS are shown. Mean \pm SE; the number of species is indicated in each bar; $n = 10$ replicate plants by species and treatment; d.w., dry weight.

Figure 3 Inducibility of (a–b) non-volatile resin and (c–d) total phenolics in the (a,c) stem and (b,d) needles of 17 Palaeartic and Nearctic pine species in response to exogenous application of methyl jasmonate (MJ), eliciting the jasmonic acid signalling defensive pathway. Inducibility is the difference in concentration between MJ-treated plants and control plants 15 days after application. F and P -values of the effect of biogeographical region of distribution after controlling for phylogeny using PGLS are shown. Mean \pm SE; the number of species is indicated in each bar; $n = 10$ replicate plants by species and treatment; d.w., dry weight.

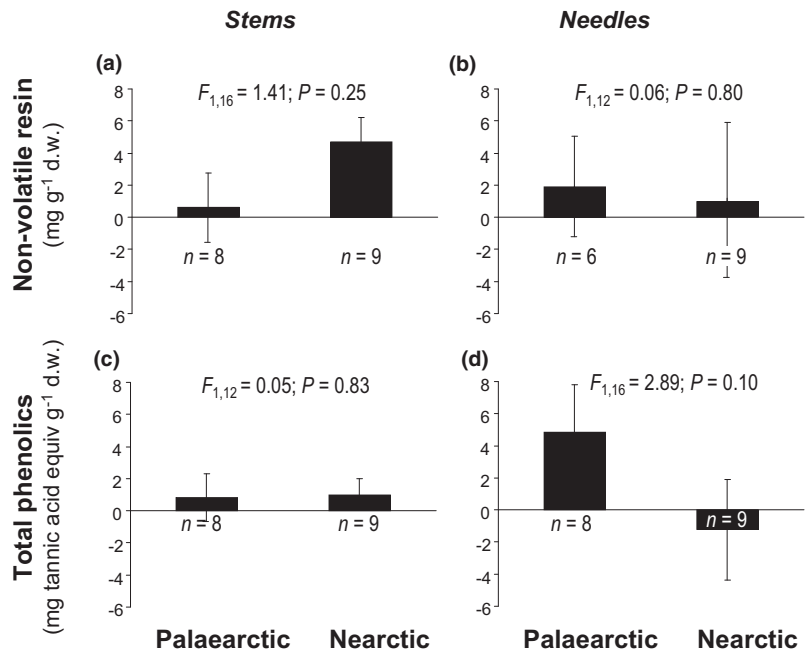
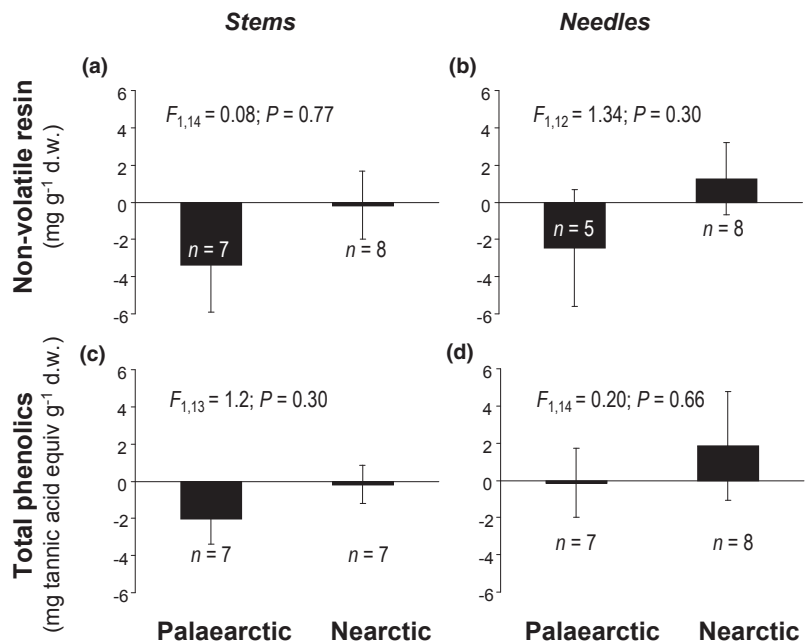


Figure 4 Inducibility of (a–b) non-volatile resin and (c–d) total phenolics in the (a,c) stem and (b,d) needles of Palaeartic and Nearctic pine species in response to exogenous application of benzothiadiazole (BTH), eliciting the salicylic acid signalling defensive pathway. Inducibility is the concentration in BTH-treated plants minus that in control plants 15 days after application. F and P -values of the effect of biogeographical region of distribution after controlling for phylogeny using PGLS are shown. Mean \pm SE; the number of species is indicated in each bar; $n = 10$ replicate plants by species and treatment; d.w., dry weight.



& Agrawal, 2011a), suggesting that herbivores are able to impose rapid evolution of plant defences, over short evolutionary time-scales (less than 100 generations), at least in herbaceous plants (e.g. Rausher & Simms, 1989; Agrawal *et al.*, 2012; Turley *et al.*, 2013). This may be the case for the induced allocation of chemical defences across pine species, in which no significant phylogenetic signal was found.

Differences in the evolution of defensive patterns across chemical defences and tissues between Nearctic and Palaeartic pine species were few, but they are consistent and important. It is particularly remarkable that Nearctic pine species invested up to 70% more in constitutive phenolics in the

needles than Palaeartic species. Leaf phenolics seem to be a recurring trait for which differences between continental ranges have emerged in several plant taxa (Lev-Yadun & Holopainen, 2009; Muilenburg *et al.*, 2011; Pearse & Hipp, 2012). Although it is well demonstrated that phenolic compounds provide resistance against herbivores and pathogens in pine trees (see revisions by Franceschi *et al.*, 2005; Mumm & Hilker, 2006), it is difficult to infer what the selective agents driving differences between ranges might be. Greater levels of this constitutive defensive trait in the Nearctic could have arisen from persistent folivory pressure (e.g. Zangerl & Rutledge, 1996), suggesting that Nearctic pines might have

shared a longer or more intense evolutionary history with defoliators than Palaeartic pines. Alternatively, current defensive traits could, for instance, be relict anachronistic adaptations resulting from past selective forces, as has been observed for other traits in long-lived trees, including autumn leaf colour and phenolic content (Lev-Yadun & Holopainen, 2009; He *et al.*, 2012). Given the strong covariation between phylogeny and biogeography in subgenus *Pinus*, the observed differences could be due to phylogenetic and biogeographical effects that cannot be disentangled. It is thus likely that the observed results are due to a mixed contribution of differing biogeographical history within each region and phylogenetic effects resulting from the early split into Nearctic and Palaeartic species. In this sense, the context of our biological system resembles that of the classic studies by Steinberg and Estes on kelp defences and herbivory along Pacific coasts (e.g. Steinberg *et al.*, 1995).

In contrast, we found no consistent biogeographical pattern in the inducibility of chemical defences, which were also found to be highly evolutionarily labile, presenting little phylogenetic signal. Analysing the data before using PGLS (Appendix S2) showed that the inducibility of stem non-volatile resin in Nearctic species in response to MJ was twice as intense as that observed for Palaeartic pines, and the inducibility of needle phenolics was much greater in Palaeartic pines than in Nearctic pines. However, these phenotypic differences were no longer significant when corrected by phylogenetic relationships by using PGLS. Based on the relevance of resin-based induced defences to the survival of bark beetles and fungi (Hudgins *et al.*, 2004; Zhao *et al.*, 2011; Schiebe *et al.*, 2012), and on longer or more sustained periods of biotic interactions with their antagonists in the Nearctic range (Lev-Yadun & Holopainen, 2009), our a priori expectation was to find greater JA-inducibility in the Nearctic.

On the other hand, pine species did not differ between ranges in the constitutive levels of non-volatile resin, nor in the inducibility of total phenolics or resin in stem and needles, suggesting that the evolution of all these defensive traits may depend more on local than on regional environmental conditions. The presence of herbivores with similar feeding guilds across distribution ranges could also lead to similar sets of defensive traits, as suggested in a study of 24 species of milkweed (Agrawal & Fishbein, 2006). It is nevertheless clear that not all pine species are subject to herbivory by all possible insect species, and levels of attack will presumably vary considerably depending on the identity of both enemy and plant host. There are several examples of the importance of biogeographical origin on constitutive or inducible plant traits of anti-herbivore resistance (e.g. Steinberg *et al.*, 1995; Desurmont *et al.*, 2011). These studies have shown that the presence or absence of a shared evolutionary history between plants and specific herbivores can lead to large differences between plant species in their defensive arsenal. In two previous studies with nine pine species, however, the inducibility of chemical defences in response to herbivory by two European insects, a bark-chewing weevil and a folivor-

ous caterpillar, did not differ substantially between European and non-European pine species (Carrillo-Gavilán *et al.*, 2012; Moreira *et al.*, 2013b). In these cases, the lack of a shared evolutionary history between the pine species and their herbivores did not explain the differences in the inducibility of chemical defences of young pine trees, suggesting that strategies based on induced defences could be defined by the evolutionary trajectories of each species with its own enemies and environmental constraints.

In summary, we found a moderately well-preserved phylogenetic signal for the constitutive concentration of defences, but not for their inducibility, except for SA-induced depletion of non-volatile resin in the stem. The strength of the phylogenetic signal in pine defences depends not only on the defensive strategy, but also on the nature of the chemical defence and plant tissue. Our results suggest that the continental scale is a good predictor of constitutive investment in needle phenolics but not for other pine defensive traits. There are no significant differences between Nearctic and Palaeartic pines in the quantitative inducibility of chemical defences, which appear to be conspicuously independent of phylogeny. Taken together, our findings suggest that the evolution of allocation to constitutive chemical defences in pine species has been more influenced by macroevolutionary history (phylogeny and past demography, climatic and biotic context in each biogeographical region) than the evolution of the inducibility of those defences. Previous results indicated that climate, as a proxy of resource availability, helps to explain patterns of pine defences (Moreira *et al.*, 2014), and here we show that biogeography, and underlying phylogenetic relationships, are also important for understanding the complex scenario of defensive allocation to constitutive and induced chemical defences among pine species.

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The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Biogeographical and phylogenetic information.

Appendix S2 Constitutive and induced concentration of quantitative chemical defences in stem and needles.

Appendix S3 Concentration of quantitative chemical defences in stem and needles in Palaeartic and Nearctic species before phylogenetic independent contrasts.

BIOSKETCH

Amparo Carillo-Gavilán is interested in the ecology of biological invasions, particularly in the possible role of plant responses in explaining the invasiveness of tree species. The experiment leading to this paper was performed at the facilities of the research group ‘Genetics and ecology of resistance to herbivores and pathogens in pine trees’ (<http://genecol.pines.weebly.com/>) at MBG-CSIC, as part of a collaborative project with EBD-CSIC (<http://www.montsevila.org/>).

Author contributions: L.S. and R.Z. conceived the ideas; A.C.-G., X.M., R.Z. and L.S. performed the experiment and collected the data; X.M., L.S. and A.C.-G. performed the chemical analyses; A.C.-G., R.Z. and A.G.-V. performed the statistical analyses; L.S., R.Z., and M.V. contributed facilities, reagents, materials, analytical tools, assistant personnel and salaries; X.M. and A.C.-G. led the writing, sharing first authorship, and wrote an initial draft; all authors contributed actively to the writing and reviewing of all versions of the manuscript.

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