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Genus-wide variation in foliar polyphenolics in eucalypts

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ABSTRACT

Many studies quantify total phenolics or total tannins, but understanding the ecological role of polyphenolic secondary metabolites requires at least an understanding of the diversity of phenolic groups present. We used UPLC-MS/MS to measure concentrations of different polyphenol groups - including the four most common tannin groups, the three most common flavonoid groups, and quinic acid derivatives - in foliage from 628 eucalypts from the genera *Eucalyptus*, *Angophora* and *Corymbia*. We also tested for phylogenetic signal in each of the phenolic groups. Many eucalypts contained high concentrations of polyphenols, particularly ellagitannins, which have been relatively poorly studied, but may possess strong oxidative activity. Because the biosynthetic pathways of many phenolic compounds share either precursors or enzymes, we found negative correlations between the concentrations of several of the constituents that we measured, including proanthocyanidins (PAs) and hydrolysable tannins (HTs), HTs and flavonol derivatives, and HTs and quinic acid derivatives. We observed moderate phylogenetic signal in all polyphenol constituents, apart from the concentration of the prodelphinidin subunit of PAs and the mean degree of polymerisation of PAs. These two traits, which have previously been shown to be important in determining plants' protein precipitation capacity, may have evolved under selection, perhaps in response to climate or herbivore pressure. Hence, the signature of evolutionary history appears to have been erased for these traits. This study is an important step in moving away from analysing "totals" to a better understanding of how phylogenetic effects influence phenolic composition, and how this in turn influences ecological processes.

1. Introduction

Polyphenolic secondary metabolites are proposed to play diverse roles mediating biotic and abiotic stress in plants, including cold tolerance (Dunning et al., 1994; Pennycooke et al., 2005), providing protection from UV damage (Lattanzio et al., 2008; Ryan et al., 2002), metal toxicity (Tahara et al., 2013) and pathogens (Lattanzio et al., 2008), and deterring insect (Barbehenn et al., 2009) and mammalian herbivores from feeding (Barthelmess, 2001; Marsh et al., 2003a). However, a full appreciation of their ecological role is limited by our capacity to adequately characterise and quantify plant polyphenols, particularly tannins.

Tannins are extremely widespread in plants, and can occur in high concentrations (Barbehenn and Constabel, 2011). There are several hundred known tannins, making the quantification of all individual tannins in plants tedious (Nierop et al., 2006; Salminen and Karonen, 2011). In terrestrial plants, tannins are usually classified as either hydrolysable tannins (HTs) or condensed tannins (proanthocyanidins; PAs). HTs are divided into two main subgroups: gallic acid derivatives (mainly galloyl glucoses and gallotannins) and ellagitannins (containing hexahydroxydiphenoyl, HHDP, groups; Fig. 1). While most simple HTs typically contain a central glucose moiety with galloyl and HHDP groups, the diversity of structures is increased by, for example, HHDP modifications and

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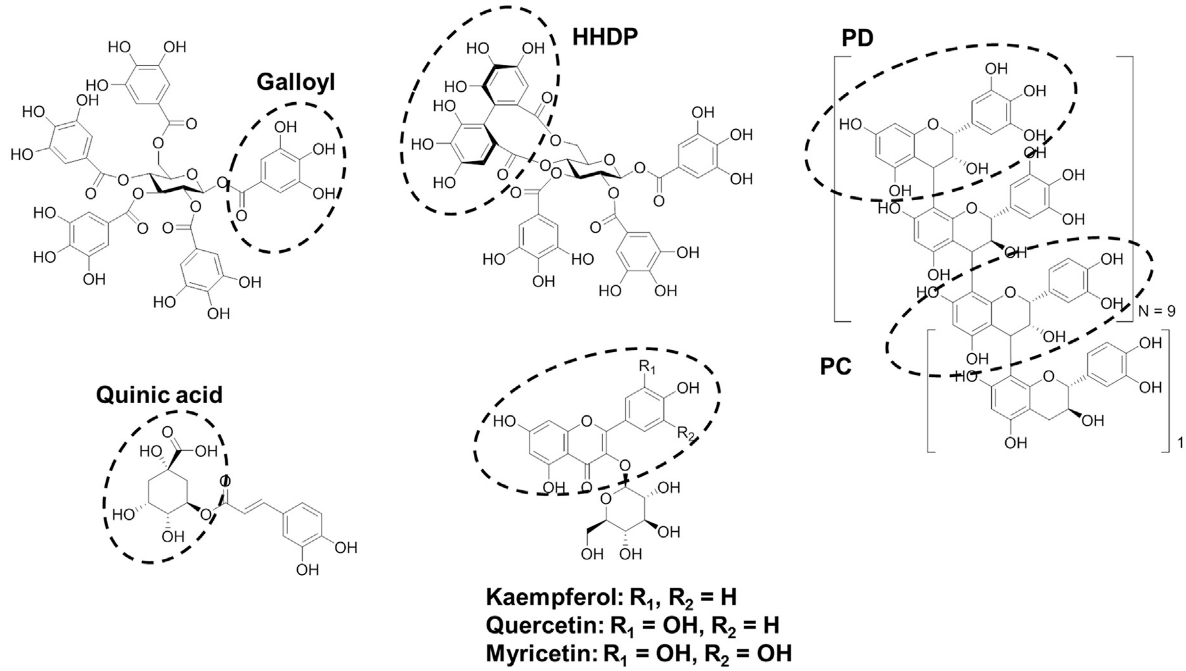

Fig. 1. Examples of polyphenol structures containing the functional groups that were measured in this study: galloyl groups, hexahydroxydiphenoyl (HHDP) groups, prodelphinidin (PD), procyanidin (PC), quinic acid, kaempferol, quercetin and myricetin.

oligomerization of the monomers, even up to undecamers (Salminen et al., 2011). In contrast, PAs are composed of monomeric flavan-3-ol units: oligomerization of catechins and gallocatechins leads to procyanidin (PC) and prodelphinidin (PD) units, respectively, in the PAs (Fig. 1). Other PA monomeric units such as properlagonidins (PP) are possible, but much less abundant.

The biosynthesis of both HTs and PAs involves the shikimate pathway, while PAs also utilise the acetate/malonate biosynthetic pathway (Salminen and Karonen, 2011). Thus, the production of large concentrations of HTs can directly reduce the production of PAs and *vice versa*, due to competition for precursors (Ossipov et al., 2004; Salminen and Karonen, 2011). Interestingly, PAs (flavonoid oligo and polymers) may also compete for precursors with flavonoid glycosides, such as kaempferol, quercetin and myricetin derivatives (Fig. 1), that are likewise made by a combination of the shikimate and acetate/malonate pathways (Johnson et al., 2014). In addition, competition between HTs and quinic acid derivatives (e.g. caffeoyl quinic acid in Fig. 1) may occur, since quinic acid shares the same precursor with gallic acid in the shikimate pathway (Ossipov et al., 2003).

Our interest in variation in polyphenol composition arises from the fact that the structures of tannins have important effects on their biological activity. For example, larger PAs are generally more effective protein precipitants than are smaller PAs (Huang et al., 2011; Kumar and Horigome, 1986; Porter and Woodruffe, 1984; Saminathan et al., 2014). The same is true for PAs containing a higher percentage of prodelphinidin, relative to procyanidin, subunits (Aerts et al., 1999). Protein-tannin complexes can negatively affect dietary protein digestibility for mammalian herbivores (Marsh et al., 2003a; Robbins et al., 1987), which has broad-reaching effects on the reproductive success of free-ranging herbivores (DeGabriel et al., 2009; McArt et al., 2009). In contrast, HTs, particularly ellagitannins, may oxidise more readily than PAs (Barbehenn and Constabel, 2011; Barbehenn et al., 2006a, 2006b). The oxidative capacity of polyphenols is recognised as a potential defence against insect herbivory (Appel, 1993; Barbehenn et al., 2005; Salminen and Karonen, 2011). As a consequence, plants with the same apparent total tannin concentration can have vastly different biological activities, which could, in turn, have important ecological consequences. Understanding the composition of tannins in plants therefore provides biological information that measures of total tannin concentrations cannot provide. Although these limitations are widely recognised, non-specific measurements of total phenolic or total condensed tannin concentrations still predominate in ecological research (e.g. Abdala-Roberts et al., 2014; Chomel et al., 2016; Couture et al., 2016; Masette et al., 2015; Volf et al., 2015; Wang et al., 2014).

Eucalypts are the dominant trees in Australia, incorporating around 900 species (Bayly et al., 2013). Eucalypt foliage contains high concentrations of phenolic secondary metabolites, particularly tannins (Fox and Macauley, 1977; Hillis, 1966), which have been implicated in protection from photo damage (Close et al., 2003; Close and McArthur, 2002), and defence against mammalian herbivores (DeGabriel et al., 2009; Foley and Hume, 1987; Marsh et al., 2003a, 2003b; Windley et al., 2016). In a comprehensive series of studies, Hillis (1966; 1967a; b; c; d) found evidence for chemotaxonomic variation in the polyphenol composition of leaves from species belonging to the genus *Eucalyptus*. Modern analytical techniques, and the availability of a well resolved eucalypt phylogenetic tree containing 711 species from the genera *Angophora*, *Corymbia* and *Eucalyptus* (Gonzalez-Orozco et al., 2016), provides an ideal opportunity to examine the evolution of variation in patterns of polyphenols in eucalypts. Recent phylogenetic analysis supports their close taxonomic affinity (Bayly et al., 2013), so we considered *Angophora* and *Corymbia* as taxonomic clades within the eucalypts.

We used UPLC-MS/MS to define the broad foliar polyphenol composition (the eight polyphenol groups shown in Fig. 1; measures are explained in Table S1) of 515 species of eucalypts. The purpose of our study was threefold: Firstly, to broadly characterise the foliar polyphenol composition of a large range of species reflecting the diversity of eucalypts, and to describe the mean