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Research

Evolutionary history explains foliar spectral differences between arbuscular and ectomycorrhizal plant species

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Summary

- Leaf spectra are integrated foliar phenotypes that capture a range of traits and can provide insight into ecological processes. Leaf traits, and therefore leaf spectra, may reflect belowground processes such as mycorrhizal associations. However, evidence for the relationship between leaf traits and mycorrhizal association is mixed, and few studies account for shared evolutionary history.
- We conduct partial least squares discriminant analysis to assess the ability of spectra to predict mycorrhizal type. We model the evolution of leaf spectra for 92 vascular plant species and use phylogenetic comparative methods to assess differences in spectral properties between arbuscular mycorrhizal and ectomycorrhizal plant species.
- Partial least squares discriminant analysis classified spectra by mycorrhizal type with 90% (arbuscular) and 85% (ectomycorrhizal) accuracy. Univariate models of principal components identified multiple spectral optima corresponding with mycorrhizal type due to the close relationship between mycorrhizal type and phylogeny. Importantly, we found that spectra of arbuscular mycorrhizal and ectomycorrhizal species do not statistically differ from each other after accounting for phylogeny.
- While mycorrhizal type can be predicted from spectra, enabling the use of spectra to identify belowground traits using remote sensing, this is due to evolutionary history and not because of fundamental differences in leaf spectra due to mycorrhizal type.

Introduction

Spectranomics is an emerging field of research that links the spectral-optical properties of leaves with plant trait and species diversity (Asner & Martin, 2009, 2016; Ustin & Gamon, 2010). Leaf traits, such as pigment or nutrient concentrations, influence how light is absorbed, reflected, and transmitted through the leaf, resulting in distinctive spectra (Gates *et al.*, 1965; Knipling, 1970; Curran, 1989; Ustin & Jacquemoud, 2020). Therefore, leaf spectra can be viewed as integrated foliar phenotypes, capturing a wide range of functional traits that can provide insights into key ecological processes (Kokaly *et al.*, 2009; Cavender-Bares *et al.*, 2016, 2017; Schweiger *et al.*, 2017, 2018; Wang *et al.*, 2020; Kothari & Schweiger, 2022). Because individual species exhibit distinct combination of traits, they can often be differentiated using spectra, enabling the spectral estimation of plant diversity (Clark *et al.*, 2005; Ustin & Gamon, 2010; Feret & Asner, 2013; Asner *et al.*, 2014; Cavender-Bares *et al.*, 2016; Schweiger & Laliberté, 2022). Spectranomics allows for the use of remote sensing technologies to map and monitor biodiversity at broad spatial scales and in a spatially explicit manner (Asner & Martin, 2016). By providing more comprehensive estimations of species and trait diversity, spectranomic tools could revolutionize biodiversity detection and monitoring. Linking leaf-level spectra with broader ecological processes at regional scales and in an evolutionary context will also improve our understanding of how biodiversity has evolved and is responding to a changing climate.

It has been hypothesized that leaf traits, and therefore leaf spectra, can reflect belowground processes such as mycorrhizal associations (Wardle *et al.*, 2004; Madritch *et al.*, 2014, 2020; Fisher *et al.*, 2016; Sousa *et al.*, 2021; Cavender-Bares *et al.*, 2022). Mycorrhizal associations can influence nutrient uptake (Tedersoo & Bahram, 2019; Chen *et al.*, 2021), foliar nitrogen levels (Craine *et al.*, 2009), nitrogen and phosphorus economic

strategies (Averill *et al.*, 2019), and other leaf economic spectrum traits (Shi *et al.*, 2020). Species with different mycorrhizal types may exhibit specific trait combinations or syndromes due to the impact of this symbiotic relationship on nutrient availability and uptake (Read, 1991; Cornelissen *et al.*, 2001). Studies have highlighted differences in leaf traits between species with arbuscular mycorrhizal (AM), ectomycorrhizal (EM), ericoid (ErM), and/or nonmycorrhizal (NM) strategies (Cornelissen *et al.*, 2001; Hayes *et al.*, 2014; Averill *et al.*, 2019). This relationship between mycorrhizal type and leaf traits contributes to the remote sensing of belowground plant traits via the spectral properties of canopies (Madritch *et al.*, 2014, 2020; Cavender-Bares *et al.*, 2022). Recent studies have demonstrated that remotely sensed spectra can discriminate between AM and EM tree species in temperate North American forests and two Hawaiian forest sites (Fisher *et al.*, 2016; Sousa *et al.*, 2021).

The relationship between mycorrhizal type and leaf spectra may be influenced by other traits such as growth form or leaf persistence. Functional groups, such as growth forms (i.e. woody, forb, and graminoid) and leaf persistence types (evergreen vs deciduous), exhibit differences in leaf traits within mycorrhizal types (Cornelissen *et al.*, 2001). These functional groups may experience different evolutionary pressures due to different approaches to resource allocation and growth and reproductive strategies that may be reflected in leaf traits and, therefore, leaf spectra (Ackerly, 2009; Flores *et al.*, 2014). For instance, when comparing annuals (typically herbaceous plants) to deciduous and evergreen woody plants, annuals tend to have higher growth rates, higher rates of carbon uptake, and have low mass per unit area, while deciduous woody plants and evergreen woody plants have successively lower growth rates, and lower rates of carbon uptake and higher leaf mass per area (Wright *et al.*, 2004; Díaz *et al.*, 2013; Reich, 2014; Ustin & Jacquemoud, 2020). Therefore, investigations of the impact of mycorrhizal type on leaf traits should factor in these functional group variables. Additionally, given that in our dataset, conifer species are predominantly EM while angiosperms are more likely to be AM, with few origins of the EM mycorrhizal type within angiosperms, this conifer–angiosperm split may also impact the observed relationship between leaf spectra and mycorrhizal type.

When comparing traits between species, it is important to account for the statistical nonindependence of trait data due to shared evolutionary history, as explained by Felsenstein (1985). Because species are related through the process of evolution, closely related species tend to have more similar traits than more distantly related species. As a result, traits measured for one species are not independent of traits measured for another so species should not be treated as if they were sampled independently from the same distribution (Felsenstein, 1985; Uyeda *et al.*, 2018). Because this nonindependence can impact the significance of trait relationships, studies which do not account for phylogenetic relatedness may overstate trait correlations or differences between groups, or misattribute phylogenetic correlation with a biological mechanism. In other words, ignoring phylogeny can result in type I errors, or false positives, where apparent support for a correlation, for example, would be due to this violation of statistical assumptions. As an example of this phenomenon, while two studies that did not account for phylogeny found significant differences in leaf traits between mycorrhizal types (Cornelissen *et al.*, 2001; Hayes *et al.*, 2014), a third study that did account for phylogeny found no significant relationship between leaf nutrients and mycorrhizal type (EM vs non-EM; Koele *et al.*, 2012). This suggests that there is a strong phylogenetic influence on the significance of this relationship and that non-phylogenetic studies may be overestimating the significance of differences in leaf traits between these mycorrhizal types (but see Averill *et al.*, 2019). In other words, studies that observe significant relationships between leaf traits and mycorrhizal type without accounting for phylogeny may be misattributing trait variation associated with shared evolutionary history to mycorrhizal type. Furthermore, if there is no significant relationship between leaf traits and mycorrhizal type after accounting for phylogeny, then any differences in leaf traits between mycorrhizal types detected by other methods (e.g. partial least squares discriminant analysis (PLS-DA)) may be the result of other factors linked to phylogeny.

Over the past 40 yr, phylogenetic comparative methods (PCM) have been developed and applied to answer evolutionary questions while accounting for phylogenetic correlation (Felsenstein, 1985; Blomberg & Garland, 2002; Blomberg *et al.*, 2003; Butler & King, 2004; Beaulieu & O'Meara, 2014; Cornwell & Nakagawa, 2017). However, until recently, application of these methods to high-dimensional multivariate traits, such as spectra, had been limited (Adams & Collyer, 2018; Clavel & Morlon, 2020). New methods implement PCM in a penalized likelihood framework (rather than a maximum likelihood framework) that allows for the application of these methods to highly dimensional data where the number of traits exceeds the number of taxa (Clavel *et al.*, 2019; Clavel & Morlon, 2020). Spectra, which are highly complex, multidimensional data, are also affected by autocorrelation, where reflectance values are correlated among neighboring wavelengths. These new methods, originally developed to analyze morphometric data (Clavel *et al.*, 2019; Clavel & Morlon, 2020; Olivares *et al.*, 2020; Reich *et al.*, 2020; Artuso *et al.*, 2021; Bardua *et al.*, 2021), are well-suited to dealing with this issue of autocorrelation and allow for testing different evolutionary models. The default model for PCM is often assumed to be Brownian Motion (BM), where trait values vary randomly along the phylogeny (Felsenstein, 1985). However, other models may be better approximations of the evolutionary history of spectra; for example, Meireles *et al.* (2020) identified the Ornstein–Uhlenbeck (OU) model, where trait values are pulled toward an optimum by selective forces, as the model that best fits spectral data.

In this study, we assess whether leaf spectra can be used to accurately predict mycorrhizal type, and whether spectral variation is associated with different mycorrhizal types. We focus on AM and EM species, which represent the majority of species both globally and in our spectral dataset (Tedersoo, 2017). We test the hypothesis that AM and EM species will differ in their spectral characteristics due to the relationships between mycorrhiza and

leaf nutrients, and between leaf nutrients and leaf spectra. Specifically, we ask: can leaf spectra be used to predict mycorrhizal type?; can evolutionary modeling of spectra reveal selective pressures on traits underlying spectra of AM and EM species?; and, are there significant differences in leaf spectra between AM and EM plant species when accounting for phylogeny?

Materials and Methods

Spectral data

Spectral data were collected by the Canadian Airborne Biodiversity Observatory (CABO) between 2018 and 2019 (Supporting Information Table S1). Sampling locations were concentrated in southern Quebec and Ontario, and coastal British Columbia (see Tables S2, S3 for details of species and locations). Spectral measurements were conducted as described by Laliberté & Soffer (2018a,b). Directional–hemispherical reflectance spectra were measured using an HR-1024i spectroradiometer with a DC-R/T integrating sphere from Spectra Vista Corp. (SVC, Poughkeepsie, NY, USA) with measurements calibrated against a white Spectralon 99% reflectance panel (Labsphere, North Sutton, NH, USA) and corrected for stray light. Spectra were measured for the adaxial surface of six sunlit leaves per individual in the field. For plants with leaves too small or narrow (e.g. needles), single-layer leaf arrays were used instead (Noda *et al.*, 2013; Laliberté & Soffer, 2018b). Healthy and intact leaves were selected, avoiding leaves with herbivore or pathogen damage.

Reflectance spectra were processed in R using the package SPECTROLAB v.0.0.10 (Meireles *et al.*, 2017) following the protocol by Schweiger & Laliberté (2020). We used linear interpolation to resample spectra to 1-nm resolution and interpolate across the region of overlap between sensors. Spectra were averaged to give a single mean spectrum per individual. A Savitzky–Golay filter (Table S4) was applied to spectra to reduce noise using the package SIGNAL 0.7.6 (Signal Developers, 2013), and spectra were trimmed to remove noisier wavelengths resulting in final spectra from 400 to 2400 nm.

The final spectral dataset contained 92 species from 27 families of vascular plants matching taxa from the trait datasets, including mycorrhizal type, and the reconstructed phylogeny. Taxonomy corresponds to the Database of Vascular Plants of Canada (VASCAN; Brouillet *et al.*, 2010; Desmet & Brouillet, 2013). The number of spectral samples vary by species, ranging between one and 120 spectral samples per species (mean = 17.93, SD = 21.33). While not all species were sampled from multiple locations, species with samples from multiple locations are spread across the phylogeny (Fig. S1) and include both mycorrhizal types (Fig. S2). Additionally, most locations included both AM and EM species (Fig. S3). The impact of intraspecific variation due to environmental conditions was also expected to be much smaller than interspecific variation (Asner *et al.*, 2014), as found in a subset of our dataset (Beauchamp-Rioux, 2022). Full spectra (wavelengths 400–2400 nm) were used for analyses unless otherwise specified (e.g. spectral regions for phylogenetic signal, or principal components of spectra for univariate models). Mean spectral reflectance for each species was used for the multivariate and univariate spectral modeling analyses, while spectra from individual samples were used for the PLS-DA.

Phylogenies

Phylogenetic analyses were conducted in BEAST v.2.6.3 (Drummond *et al.*, 2012; Bouckaert *et al.*, 2019) to generate a distribution of phylogenies from which 100 trees were randomly selected for analysis. A maximum clade credibility (MCC) tree was generated from the posterior distribution of trees. Each phylogeny was pruned to 92 species to match the trait and spectral dataset. A full description of the methods used to generate these phylogenies is available in Methods S1, Tables S5–S7 and Figs S4 and S5.

Predictor data

Species-level mycorrhizal types were retrieved from the FUNGALROOT database (Soudzilovskaia *et al.*, 2020) and additional published sources (Table S8). The most probable primary mycorrhizal type was assigned based on species-level root observations. Conflicting data on mycorrhizal type at the species level were resolved based on expert knowledge (Soudzilovskaia *et al.*, 2020). When information was not available at the species level, the mycorrhizal type was inferred from a higher taxonomic level (i.e. genus, or in the case of *Oemleria cerasiformis* (Torrey & A. Gray ex Hooker & Arnott) J.W. Landon, family) following Soudzilovskaia *et al.* (2020). Each species was scored as having arbuscular (AM), ericoid (ErM), ectomycorrhizal (EM), dual AM–EM, or no documented (NM) mycorrhizal association. We excluded species with ErM (three species) and dual AM–EM (three species) mycorrhizal types and those scored as NM (one species) due to the few species with each of these types preventing informative statistical analysis, resulting in 92 species with mycorrhizal data. Growth form (i.e. habit) and taxonomic data were retrieved from VASCAN (Brouillet *et al.*, 2010; Desmet & Brouillet, 2013) for each species using the *vascan_search* function in the R package TAXIZE (Chamberlain & Szocs, 2013; Chamberlain *et al.*, 2020). Growth form was encoded as a three-state categorical variable (tree, shrub, and herb). Taxonomic information was parsed, and four taxonomic levels were retained: class, subclass, superorder, and order. Leaf persistence data (binary variable, evergreen vs deciduous) were obtained from the TOPIC database (Aubin *et al.*, 2012). For species not represented in the TOPIC database, trait values were compiled from electronic floras and databases (Flora of North America Editorial Committee, 2012; Klinkenberg, 2021; USDA & NRCS, 2022).

Leaf trait data

Five leaf traits were measured for 91 species: nitrogen and lignin content (per unit mass), and Chl*a* and Chl*b*, and carotenoid content (per unit area) according to published protocols (Ayotte *et al.*, 2018; Ayotte & Laliberté, 2019; Girard *et al.*, 2020). Leaf mass per area (LMA) and equivalent water thickness (EWT) were

measured for all 92 species (Laliberté, 2018; Schweiger *et al.*, 2020; Kothari *et al.*, 2022).

Partial least squares discriminant analysis

Partial least squares discriminant analysis was conducted in R v.3.6.1 to determine whether spectra from AM species can be discriminated from spectra of EM species using the packages CARET, STATS, and AGRICOLAE (R Core Team, 2019; Kuhn, 2020; de Mendiburu, 2021; Wei & Simko, 2021). Partial least squares discriminant analysis was conducted with the full dataset (92 species) and with only angiosperms (80 species). Individual spectral samples were split into training and testing datasets (75% and 25%, respectively, for each mycorrhizal type). The number of spectral components used was determined based on significant differences in kappa statistics as determined by the Tukey's test (Figs S6, S7; Schweiger, 2022); we ran 100 iterations for 19 components for all 92 taxa, and 20 components for angiosperms only.

Phylogenetic signal of predictors and spectra

The distributions of trait values for the predictor variables (mycorrhizal type, growth form, and leaf persistence) across the phylogeny were visualized using the MCC tree. For binary traits (leaf persistence and mycorrhizal type), phylogenetic signal was measured as the phylogenetic *D* statistic, as calculated using the *phylo.d* function in the R package CAPER (Orme *et al.*, 2018). Phylogenetic signal for the categorical variable (growth form) was measured as delta, as described and coded by Borges *et al.* (2018).

Phylogenetic signal was calculated for spectral data using the multivariate metric of Blomberg's *K*, K_{multi}, using GEOMORPH v.4.0.0 (Blomberg *et al.*, 2003; Adams & Otárola-Castillo, 2013; Adams, 2014; Baken *et al.*, 2021; Adams *et al.*, 2022), for each of the spectral regions (VIS, visible; NIR, near infrared; SWIR, shortwave infrared) and each class, subclass, superorder, and order with more than two species for each of the randomly sampled 100 phylogenetic trees. Significance was assessed from comparison with a null distribution generated from 999 permutations.

Models of spectral evolution

Multivariate models Using the R package MVMORPH (Clavel *et al.*, 2015), the evolutionary dynamics of spectra were modeled using regressions of spectra against the intercept for four evolutionary models over 100 randomly selected phylogenies. These methods, which can be applied to multivariate datasets with more traits than taxa, are implemented in a penalized likelihood framework rather than a maximum likelihood framework to ensure that the covariance matrix is symmetric-positive definite and invertible (Clavel *et al.*, 2019). The evolutionary models used to quantify the covariance in the regression include pure BM, where the covariance structure is based on shared branch lengths on the phylogeny, or models where the covariance structure is modified due to selection toward an optimum (Ornstein–Uhlenbeck (OU) model), decreasing evolutionary rate over time (Early Burst (EB) model), or other processes that may produce phylogenetic signal that deviates from pure BM (lambda model). The best fit model for each tree was determined using Generalized Information Criterion (GIC; Konishi & Kitagawa, 1996).

Univariate models Phylogenetic principal component (pPC) axes represent univariate components of spectral variation that can be modeled using more complex evolutionary models available in the R package OUWIE (Beaulieu & O'Meara, 2021). These models can identify single or multiple rates of evolution (for BM and OU evolutionary models) and single or multiple optima (OU models). Incorporating these more complex models helps tease apart different selective pressures on these components of spectral variation by identifying whether multiple rates or optima may correspond to different predictor trait states. Phylogenetic principal components analysis (pPCA), incorporating the intercept-based variance–covariance matrices, was conducted using the *mvgls.pca* function in MVMORPH (Clavel *et al.*, 2015). The top pPC axes that explained 99% of the spectral variation were retained for statistical analysis.

Univariate models were generated for each of the top three pPC axes, which represent *c.* 98% of spectral variation and appear biologically meaningful (i.e. represent interpretable spectral variation and not just noise), using the function *OUwie* with mycorrhizal state stochastically mapped on each phylogeny using the function *make.simmap* from the package PHYTOOLS (Revell, 2012; Beaulieu & O'Meara, 2021). Seven evolutionary models were fit: BM1, BMS, OU1, OUM, OUMV, OUMA, and OUMVA (Table S9); the fit of these univariate models was assessed using AIC (Akaike, 1974). To assess reliability of parameter estimates for the univariate *OUwie* models, eigenvectors of the Hessian matrix were evaluated for negative values. The standard errors of the model parameters were also evaluated to assess the stability of parameter estimates.

Significance tests

Multivariate analyses To test the hypothesis that AM and EM species have significantly different spectra, we examined the relationship between spectra as the response variable and mycorrhizal type (MYC), growth form (GF), and leaf persistence (LP) as predictors. Growth form and leaf persistence are included as predictors in these models due to the influence that these variables may have on leaf nutrients and spectra within mycorrhizal type (Cornelissen *et al.*, 2001). In addition, we tested models including the interaction between MYC and GF or LP, respectively.

Using the R package MVMORPH (Clavel *et al.*, 2015), regression models were constructed for these combinations of predictors with the four evolutionary models (BM, OU, EB, and lambda) over the distribution of 100 randomly selected phylogenies. The best fit model for each tree was determined using GIC (Konishi & Kitagawa, 1996). Generalized Information Criterion is an unreliable metric when using multiple predictors in MVMORPH so

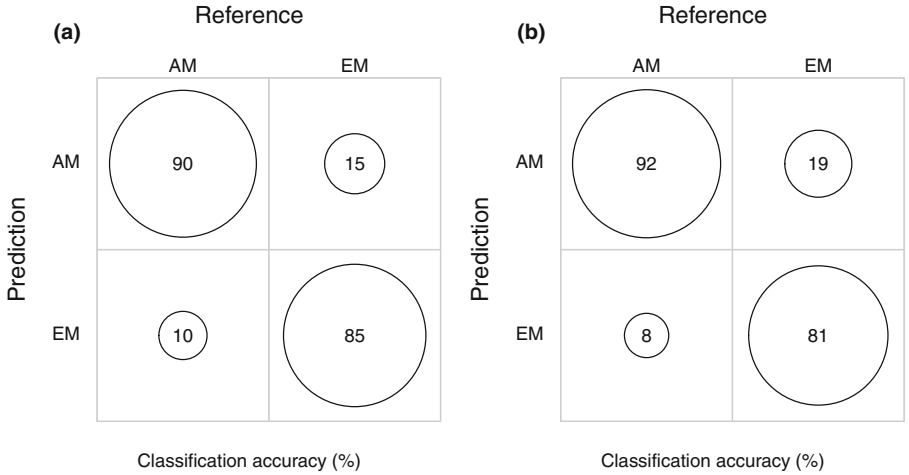

Fig. 1 Partial least squares discriminant analysis confusion matrix for arbuscular mycorrhizal (AM) and ectomycorrhizal (EM) type with percentage of samples accurately classified (diagonal) and inaccurately classified (off-diagonal) for (a) all species and (b) angiosperm species only.

model parameters were also examined to determine model fit (Clavel *et al.*, 2015). To determine whether the multivariate models violated statistical assumptions of linear regressions, the distribution of residuals and the relationship between residuals and fitted values were visualized.

Using the best fit models, multivariate analyses of variance (MANOVAs) were conducted for these predictors to determine whether spectral variation is significantly associated with mycorrhizal type, and whether growth form or leaf persistence influence the relationship between spectra and mycorrhizal type.

Univariate analyses Regular (nonphylogenetic) principal components analysis (PCA) was conducted for the spectral data using the *prcomp* function from the STATS package in R (R Core Team, 2019). The top six PC axes that explained 99% of the spectral variation were analyzed using the *manova* and *summary.aov* functions from the STATS R package (R Core Team, 2019) to assess the statistical support for differences between AM and EM species without accounting for phylogeny.

ANOVA tests were conducted using the function *aov* to test for significant differences between mycorrhizal types in pPC axes generated as described above (R Core Team, 2019). Because pPCA only phylogenetically corrects the eigenvectors, and not the scores, these ANOVA tests are not phylogenetically corrected analyses (Revell, 2009; Polly *et al.*, 2013). To account for phylogeny using these pPC axes, phylogenetic regressions against mycorrhizal type were conducted for each of the top five pPC axis and for each phylogenetic tree using the *phylolm* function in R (Tung Ho & Ané, 2014), comparing four evolutionary models (BM, lambda, EB, and OUrandomRoot); fit was assessed using AIC and the best models were retained to assess significance of the relationship between pPC axes and mycorrhizal type.

Analysis of leaf traits The empirically measured leaf traits (LMA, EWT, and nitrogen, lignin, Chl*a* and Chl*b* and carotenoid content) were analyzed for statistical differences between mycorrhizal type both with (*gls*) and without (*lm*) accounting for phylogeny (Pinheiro *et al.*, 2019; R Core Team, 2019).

Results

Classification of AM and EM species using PLS-DA

Partial least squares discriminant analysis was able to discriminate between AM and EM species with high accuracy (90% AM–AM, 85% EM–EM, 19 component model; Figs 1, S6). More EM samples were misidentified as AM (15%) than vice versa (10%). The ability of PLS-DA to discriminate between AM and EM species was maintained when only angiosperms are evaluated (92% AM–AM, 81% EM–EM, 20 component model; Figs 1, S7). When including only angiosperms, misidentifications of EM species as AM increased to 19%, while the misidentifications of AM as EM decreased (8%).

Distributions of spectral and predictor data

Arbuscular mycorrhizal species have higher average reflectance than EM species although spectral variance is high for both AM and EM species (Fig. 2). Mycorrhizal type, growth form, and leaf persistence appear phylogenetically clustered (Fig. 3). Mycorrhizal type is significantly correlated with both growth form ($\chi^2 = 27.628$, $P = 1e\text{-}06$) and leaf persistence ($\chi^2 = 5.8249$, $P = 0.0158$) according to chi-squared tests of independence. Mycorrhizal type and growth form show high levels of phylogenetic signal (Table S10), while spectra tend to show lower levels of phylogenetic signal, with a few exceptions (Table 1).

Models of spectral evolution

The intercept multivariate models identified EB as the best model for 68 phylogenetic replicates, OU as the best model for 29 replicates, and lambda as best for the remaining three phylogenetic replicates. Brownian Motion was not identified as the best fitting model for any replicate. Parameter estimates across the EB, OU, and lambda models are consistent with deviation from BM. The phylogenetic half-life for the OU models is 45.8 ± 4.87 million years (Myr) across the phylogeny with a total tree length of 345 ± 30.3 Myr (Table S11). For the EB models, the rate of

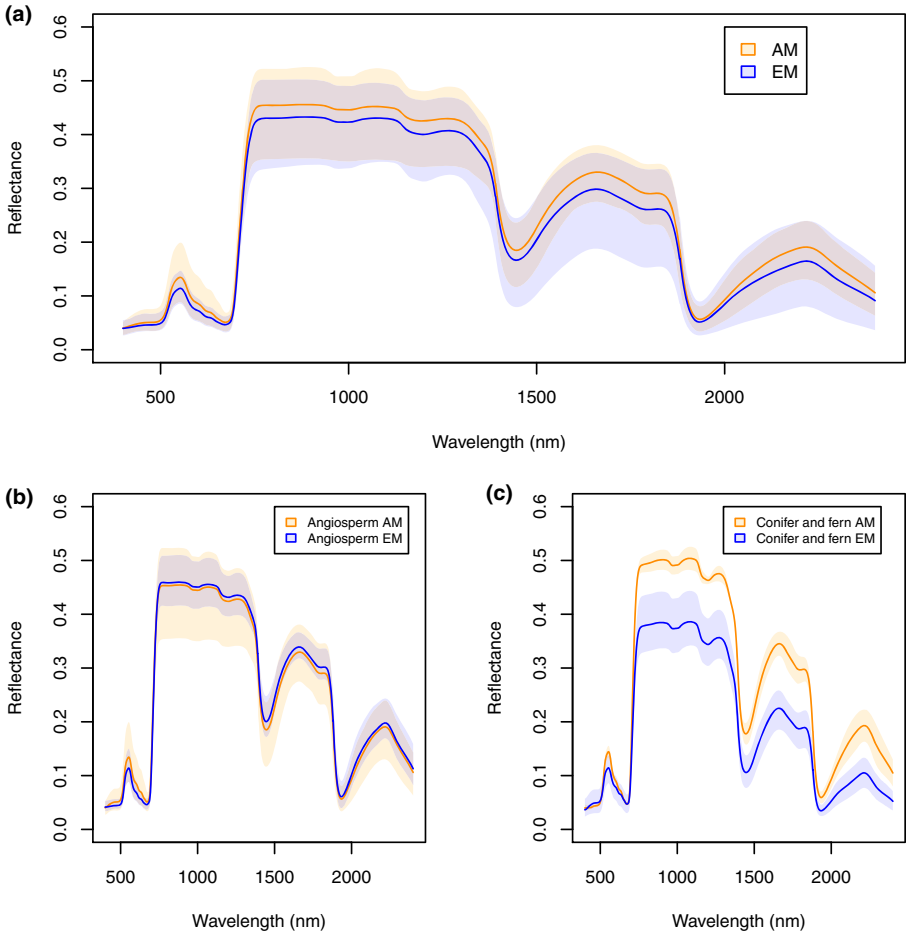

Fig. 2 Mean spectral reflectance with 95% quantile for arbuscular mycorrhizal (AM; orange) and ectomycorrhizal (EM; blue) plant species for (a) all taxa, (b) angiosperms only, and (c) conifer and fern species only (only one fern species: AM).

deceleration (r) is -0.0153 ± 0.002 while the estimate of lambda for the lambda models is $0.200 \pm 3.06e\text{-}06$ (Table S11; Fig. S8).

Evolutionary models were fit to the top three univariate pPC axes that represent $66.6 \pm 2.29\%$, $27.0 \pm 1.75\%$, and $4.46 \pm 0.428\%$ of spectral variation, respectively, and appear to have a biological interpretation in the context of spectral variation (Fig. 4b) and do not represent noise. For all three axes, BM was rejected as the best fitting model (Table S12). For each phylogenetic replicate, one of the five variants of the OU model was found to be the best fit for these three axes. Multiple optima corresponding to AM and EM species were identified for pPC 1 and pPC 3, while a single optimum was identified for most phylogenetic replicates of pPC 2 (Table S12; Figs 4, 5, S9). Only a single negative eigenvalue was recovered from the Hessian matrices of the 100 phylogenetic replicates for the top three pPC axes indicating that these models and parameters are reliable. By contrast, multiple parameters were identified as unstable based on the size of the standard error, mainly for the theta estimates for EM species; however, these parameter values were consistently reported when the analysis was repeated multiple times.

Statistical tests

Multivariate analyses Within the multivariate penalized likelihood framework, using the best fitting evolutionary models to generate the variance–covariance structure, the phylogenetic MANOVAs recovered no significant difference between spectra for AM and EM species ($P = 1 \pm 0$) for any of the phylogenetic replicates (Table 2). No significant differences in spectra between AM and EM species were observed for different growth forms, leaf persistence types, or their interactions within the multivariate PCM framework (Table 2). The distributions of residuals were approximately normal and homoskedastic; transforming the data (square root of 100 × spectral reflectance) did not improve the distribution of residuals significantly (Figs S10, S11).

Univariate analyses ANOVAs of regular PC axes showed significant differences between AM and EM species (Table 3). Significant differences between mycorrhizal types were observed when analyzing the top six PC axes together ($P = 6.04e\text{-}05$); when examining the regular PC axes independently, significant differences were observed for PC 1 ($P = 0.003$, 61.6% variance explained), PC 3 ($P = 0.011$, 12.4%), and PC 5 ($P = 0.049$, 0.9%). When the Holm correction for multiple tests was applied, only PC axis 1 was found to be significant ($P = 0.018$), while PC axis 3 almost reached our significance threshold ($P = 0.056$). Significant differences between AM and EM species were also observed for nonphylogenetic ANOVAs of phylogenetic PC axes for

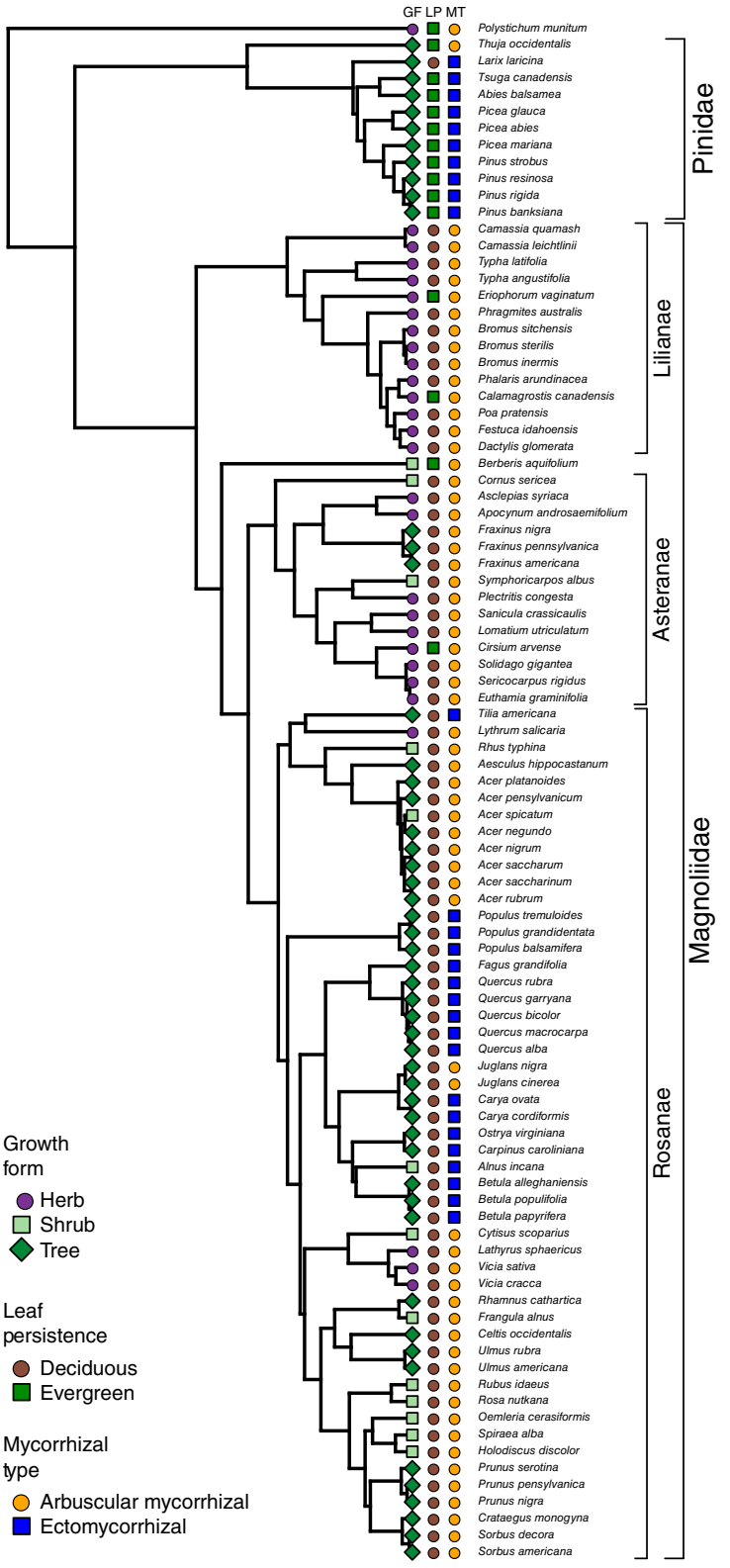

Fig. 3 Distribution of predictor variables on maximum clade credibility phylogeny for 92 species. Growth form (GF): herb (purple), shrub (light green), tree (dark green). Leaf persistence (LP): deciduous (brown), evergreen (green). Mycorrhizal type (MT): arbuscular mycorrhizal (orange), ectomycorrhizal (blue).

four pPC axes: pPC 1 ($66.6 \pm 2.3\%$ variance explained), pPC 3 ($4.46 \pm 0.43\%$), pPC 4 ($0.697 \pm 0.046\%$), and pPC 5 ($0.417 \pm 0.038\%$; Fig. 6a).

However, phylogenetic linear regression (*phylolm*) of pPC axes identified few significant differences between AM and EM species for individual pPC axes (Fig. 6b). The only pPC axis with a high

Table 1 Phylogenetic signal of spectra as measured by K_{multi}.

Taxonomic level	Clade	No. of taxa	Spectral range (nm)	K_{multi} (mean ± SD)	*P*-value (mean ± SD)	No. of significant replicates (/100)
Class	Equisetopsida	92	400–699 (VIS)	0.094 ± 0.028	0.016* ± 0.074	96
	Equisetopsida	92	700–1399 (NIR)	0.103 ± 0.026	0.007** ± 0.039	98
	Equisetopsida	92	1400–2400 (SWIR)	0.161 ± 0.033	0.001** ± 2.4e-04	100
	Equisetopsida	92	400–2400	0.123 ± 0.028	0.002** ± 0.005	100
Subclass	Pinidae	11	400–2400	**0.836** ± 0.301	0.007** ± 0.008	99
	Pinidae	11	400–699 (VIS)	0.221 ± 0.130	0.416 ± 0.155	0
	Pinidae	11	700–1399 (NIR)	**0.843** ± 0.324	0.0360* ± 0.027	82
	Pinidae	11	1400–2400 (SWIR)	**1.045** ± 0.222	0.002** ± 0.002	100
	Magnoliidae	80	400–2400	0.135 ± 0.028	0.005** ± 0.034	99
	Magnoliidae	80	400–699 (VIS)	0.185 ± 0.049	0.005* ± 0.034	99
	Magnoliidae	80	700–1399 (NIR)	0.123 ± 0.029	0.016* ± 0.074	94
	Magnoliidae	80	1400–2400 (SWIR)	0.150 ± 0.028	0.001** ± 0.004	100
Superorder	Rosanae	51	400–2400	0.131 ± 0.025	0.004** ± 0.006	100
	Asteranae	14	400–2400	0.114 ± 0.046	0.432 ± 0.177	0
	Lilianae	14	400–2400	**0.612** ± 0.109	0.001** ± 6.9e-04	100
Order	Pinales	10	400–2400	**0.556** ± 0.207	0.047* ± 0.034	61
	Sapindales	10	400–2400	0.121 ± 0.041	0.152 ± 0.064	0
	Fagales	16	400–2400	0.183 ± 0.063	0.021* ± 0.055	91
	Poales	12	400–2400	**0.669** ± 0.130	0.006** ± 0.004	100
	Rosales	16	400–2400	0.166 ± 0.042	0.658 ± 0.127	0
	Asterales	4	400–2400	0.210 ± 0.124	0.735 ± 0.180	0
	Fabales	4	400–2400	0.744 ± 0.153	0.448 ± 0.053	0
	Lamiales	3	400–2400	0.707 ± 0.242	0.226 ± 0.056	0

Bold – significant and high phylogenetic signal ($K_{multi} > 0.5$ and P-value < 0.05). Spectral regions: VIS, visible; NIR, near infrared; SWIR, shortwave infrared. Significance levels: *, $P < 0.05$; **, $P < 0.01$.

number of significant phylogenetic replicates was pPC axis 5; however, this axis appears to have no obvious biological interpretation and represents only 0.417 ± 0.0377% of spectral variation (Fig. S12).

Leaf traits Several empirically measured leaf traits were also significantly different for AM and EM species when not accounting for phylogeny; however, no significant differences between AM and EM species were found for any of the leaf traits when accounting for phylogeny (*gls*; Table 4).

Discussion

Mycorrhizal type can be accurately predicted from leaf spectra, supporting the application of machine learning methods to predict belowground traits using remotely sensed spectra, although what is actually being detected is the relationship between mycorrhizal type and phylogeny rather than intrinsic traits related to mycorrhizal type. However, using multivariate PCM, we find that when accounting for phylogeny, no significant relationship is observed between mycorrhizal type and leaf spectra. Species with AM and EM mycorrhizas are evolving toward different spectral optima on certain axes of variation, emphasizing the influence of evolutionary history on the relationship between mycorrhizal type and leaf spectra.

Leaf spectra predict mycorrhizal type

Mycorrhizal type was predicted with high accuracy using PLS-DA, which indicates that machine learning is a reliable approach for monitoring plant traits using spectra, including those only indirectly associated with spectra (Fisher *et al.*, 2016; Sousa *et al.*, 2021). Even when analyzing only angiosperms, and therefore removing any impact of the conifer–angiosperm split, PLS-DA accurately classified species' mycorrhizal type based on spectra, suggesting that this approach may be successful when applied to different contexts, and not only when species have dramatically different leaf morphology. Partial least squares discriminant analysis is an effective way of discriminating between categories even at finer scales, such as between species (Girard *et al.*, 2020; Kothari & Schweiger, 2022). Therefore, it is not surprising that spectra could discriminate larger aggregations of species such as groupings by mycorrhizal type, especially given that each species in these analyses belongs to a single type. There are key differences in the methods used and the questions answered by PLS-DA compared with PCM. Machine learning methods such as PLS-DA do not account for correlation among species and use individual-level data rather than species means, increasing the number of data points available. Partial least squares discriminant analysis can predict categories based on small differences but does not assess the significance of the relationship between mycorrhizal type and spectra. The predictive power of PLS-DA is useful for

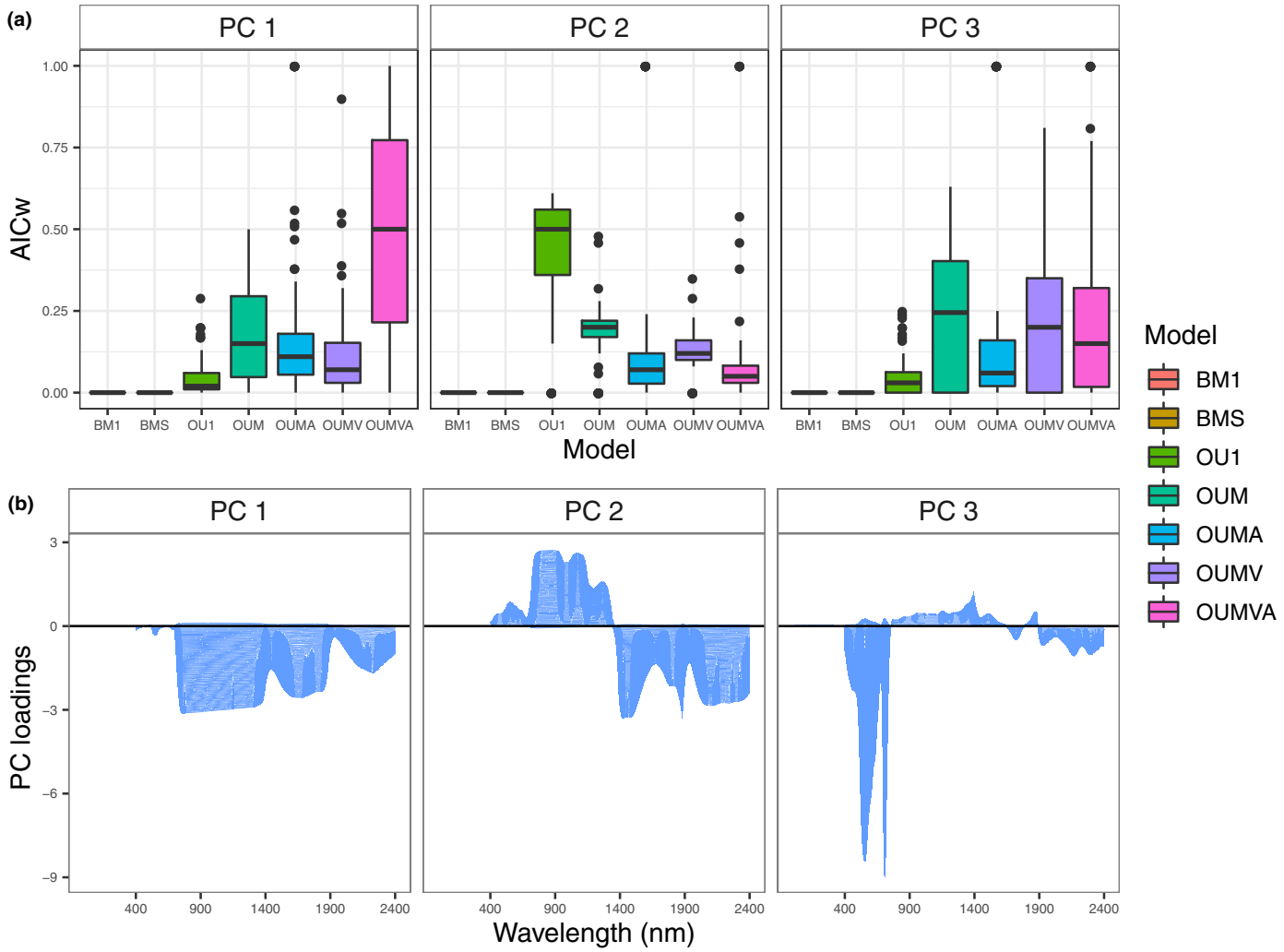

Fig. 4 Univariate modeling results of phylogenetic principal components. (a) Akaike Information Criterion weights (AICw) for univariate evolutionary models for the top three phylogenetic principal components (PC) axes for 100 phylogenetic replicates represented by boxplots with the median (horizontal line) and 25th and 75th percentiles (box boundaries) illustrated with whiskers representing values within 1.5 IQR and outliers > 1.5 IQR plotted as open circles. Best fitting evolutionary model for the most phylogenetic replicates: PC1, OUMVA; PC2, OU1; PC3, OUM (Supporting Information Table S12). Evolutionary models: BM1, single rate Brownian Motion (BM); BMS, multiple rate BM; OU1, single optimum Ornstein-Uhlenbeck (OU); OUM, multiple optimum OU; OUMA, multiple optimum and multiple alpha OU; OUMV, multiple optimum and multiple sigma^2 OU; OUMVA, multiple optimum, sigma^2, and alpha OU. (b) pPC loadings for the corresponding top three pPC axes.

many applications including remote sensing and biodiversity monitoring but does not provide insight into the evolutionary relationship between spectra and mycorrhizas. Therefore, it is important to be clear about what is being detected (i.e. groupings rather than relationships) when applying this method in different contexts and to ensure that when interpreting these classifications, accurately classifying groups is not conflated with identifying biologically meaningful traits. These methods may also be less accurate in cases where the mycorrhizal type-phylogeny relationship differs from what we observed in this study.

Evolutionary models of spectra

The relationship between traits and phylogenies can be quantified by phylogenetic signal. Mycorrhizal type is strongly associated with phylogeny and shows high phylogenetic signal (Table S10). The EM strategy has four evolutionary origins within our dataset: in conifers, Fagales, Salicaceae, and *Tilia*. This contributes to the close relationship between mycorrhizal type and phylogeny. Spectra are not as closely linked to phylogeny, except for within certain clades such as conifers and monocots, and the estimate of phylogenetic signal for multivariate spectra (Table 1) reflects a deviation from the pattern of BM. As an integrated phenotype comprising many foliar traits that are presumably under selection, it was expected that leaf spectra would deviate from pure BM. This is consistent with Meireles *et al.* (2020) who showed that certain wavelengths had low phylogenetic signal ($K < 1$) and evolved under an OU model rather than a BM model. The high dimensionality of spectra may also contribute to this lower estimate; as the K_{multi} statistic is calculated over the entire range of wavelengths, wavelengths with high phylogenetic signal may be obscured by those with low phylogenetic signal. However,

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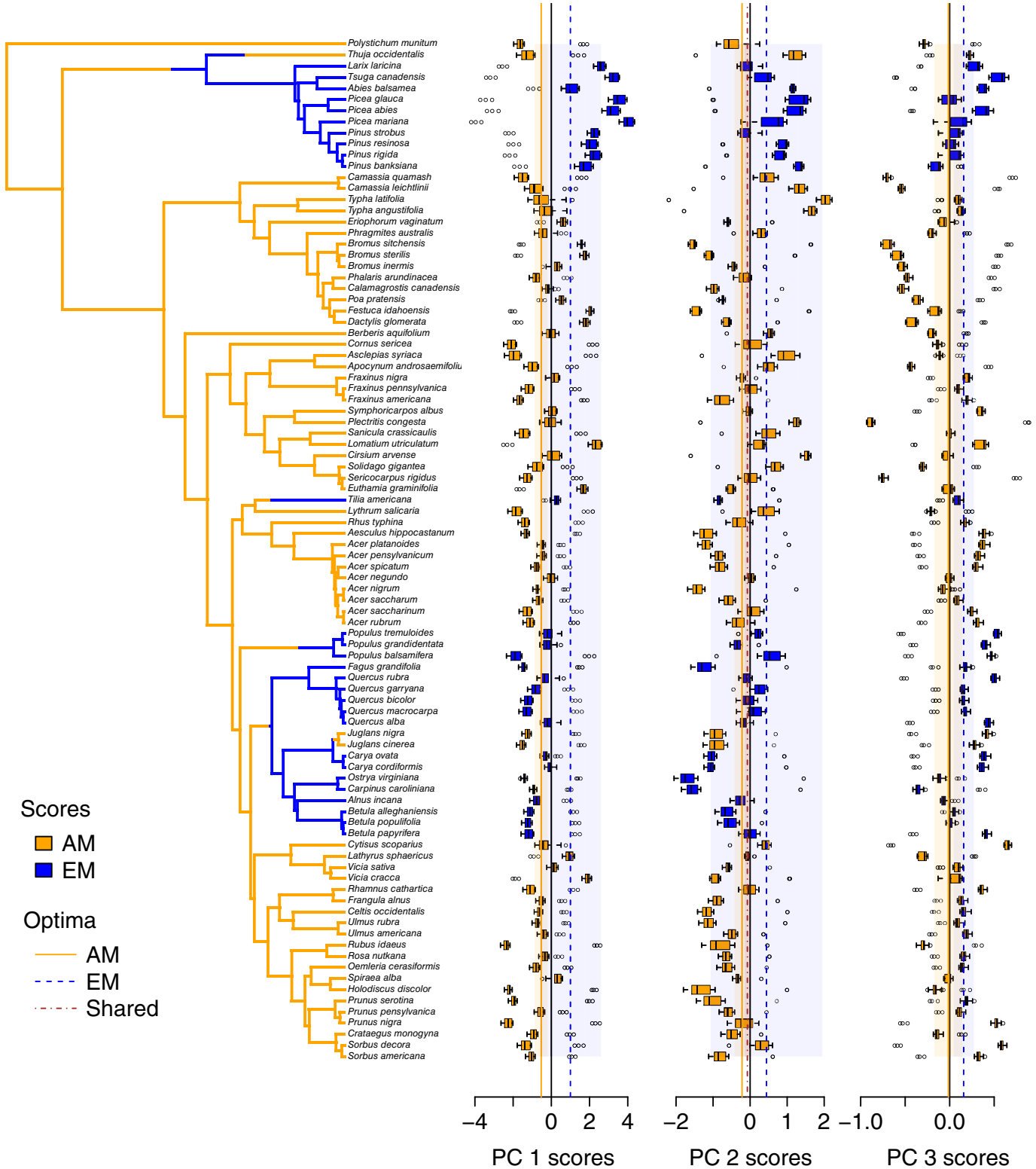

Fig. 5 Maximum clade credibility phylogeny with arbuscular mycorrhizal (AM; orange) and ectomycorrhizal (EM; blue) states stochastically mapped. Phylogenetic principal components (PC) scores for 100 phylogenetic replicates for pPC axes 1–3 are colored by mycorrhizal type (AM, orange; EM, blue) with boxplots illustrating the median (vertical line) and 25th and 75th percentiles (box boundaries), with whiskers representing values within 1.5 IQR and outliers > 1.5 IQR plotted as open circles. Optima (vertical lines) with 1 SD variation (translucent shading) are plotted for each pPC. Optima are colored by mycorrhizal type for pPCs 1 and 3 (AM, orange solid; EM, blue dashed), and by each mycorrhizal type for 19 multiple optimum Ornstein–Uhlenbeck (OUM+) models and by the shared optimum for 81 single optimum Ornstein–Uhlenbeck (OU1) models for pPC 2 (AM, orange solid; EM, blue dashed; shared, brown dot-dashed).

Table 2 Phylogenetic MANOVA results for mycorrhizal type, growth form, leaf persistence, and interactions between mycorrhizal type and growth form or leaf persistence respectively.

Predictor(s)	*P*-value (mean ± SD)	Test stat (mean ± SD)	Phylogenies significant (%)
Mycorrhizal type (AM vs EM)	1 ± 0	0.238 ± 0.011	0
Growth form (tree, shrub, herb)	0.926 ± 0.15	0.017 ± 0.021	0
Leaf persistence (evergreen, deciduous)	0.944 ± 0.044	0.129 ± 0.077	0
Mycorrhizal type × growth form	MYC: 1.0 ± 1e-05	0.568 ± 0.077	0
	GF: 0.987 ± 0.090	0.097 ± 0.030	0
	MYC : GF: 0.999 ± 0.002	0.426 ± 0.046	0
Mycorrhizal type × leaf persistence	MYC: 1.0 ± 0.002	0.469 ± 0.092	0
	GF: 0.990 ± 0.043	0.277 ± 0.068	0
	MYC : GF: 0.999 ± 0.005	0.340 ± 0.098	0

AM, arbuscular mycorrhizal; EM, ectomycorrhizal; GF, growth forn; MYC, mycorrhizal type.

Table 3 ANOVA results for the top six regular principal component (PC) axes representing 99% of spectral variation.

Predictor	PC axis	Variance explained (%)	*P*-value	*F*-value	Adjusted *P*-value (Holm correction)
Mycorrhizal type	1	61.6	0.0030**	9.289	0.0182*
	2	22.2	0.3426	0.9101	0.3426
	3	12.4	0.0112*	6.703	0.0561
	4	1.7	0.0938	2.868	0.1968
	5	0.9	0.0492*	3.976	0.1968
	6	0.3	0.0635	3.530	0.1968
	1 : 6	99.3 (cumulative)	6.037e-5**	5.614	NA

Significance levels: *, *P* value < 0.05; **, *P* value < 0.01.

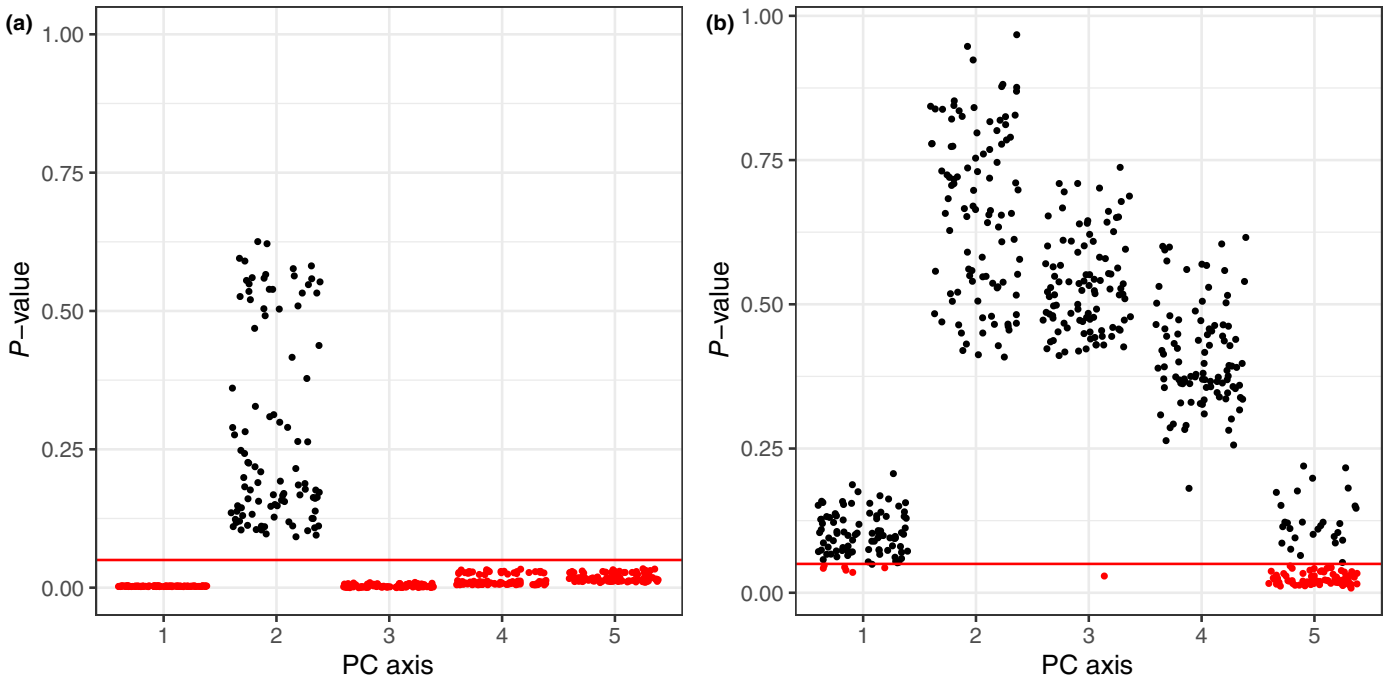

Fig. 6 *P*-values of significance tests for the top five phylogenetic principal components (PC) axes representing 99% of spectral variation and mycorrhizal type. Each point represents a phylogenetic replicate. Red line, significance threshold of 0.05. Values below the significance threshold are colored red. (a) Nonphylogenetic ANOVA. (b) Phylogenetic linear regression.

Table 4 Nonphylogenetic (*lm*) and phylogenetic regression (*gls*) results for leaf traits.

Trait (unit)	No. of taxa	Nonphylogenetic *P*-value (*lm*)	Nonphylogenetic *P*-value (*lm* with Holm correction)	Phylogenetic *P*-value (*gls*)	Phylogenetic *P*-value (*gls* with Holm correction)
LMA (g m^{-2})	92	9.68e-07***	6.78e-06***	0.415	1
EWT (μm)	92	0.0503^	0.201	0.481	1
Nitrogen (% per unit mass)	91	0.00445**	0.0267*	0.277	1
Lignin (% per unit mass)	91	0.0191*	0.0956	0.783	1
Chl*a* (% per unit area)	91	0.208	0.624	0.676	1
Chl*b* (% per unit area)	91	0.242	0.624	0.725	1
Carotenoid (% per unit area)	91	0.502	0.624	0.455	1

EWT, equivalent water thickness; LMA, leaf mass per area. Significance levels: ^, $P < 0.06$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

estimates of phylogenetic signal for the three main spectral regions (VIS, NIR, and SWIR) tend to be congruent with estimates calculated over the entire spectrum (Table 1). Consistent with lower phylogenetic signal in spectra, OU or EB evolutionary models were identified as the best fitting models for many phylogenetic replicates of the multivariate intercept spectral models, providing additional evidence for deviation from BM.

According to the univariate analysis of pPC axes, species with AM are evolving toward a different spectral optimum than species with EM, as illustrated by pPCs 1 and 3. While these results indicate an association between mycorrhizal type and spectral optima, the close link between mycorrhizal type and phylogeny may be driving this relationship. In our dataset, conifer species are almost exclusively EM while most angiosperm species are AM. We would therefore expect spectral differences across this deep phylogenetic split to be significantly correlated with mycorrhizal type, regardless of whether most spectral variation is due to mycorrhizal type or to other traits (i.e. needles vs broad leaves). While mycorrhizal type may be expected to contribute to some spectral differences between conifers and angiosperms, it is likely that other factors are playing a more dominant role in determining this spectral variation. This association between mycorrhizal type and phylogeny also occurs within the angiosperm clade, where EM species are found in few lineages, and our univariate model results are replicated when analyzing only angiosperm species (Methods S2; Table S13; Figs S13–S15). Therefore, we suggest that the association of mycorrhizal type with different spectral optima is the result of the close association of mycorrhizal type with phylogeny.

The pPC axis 1, representing 66.6 ± 2.3% of spectral variation, appears to comprise spectral variation across the near infrared (NIR) and shortwave infrared (SWIR) regions, wavelengths that are typically associated with water content, structural elements including mesophyll structure and LMA, and biochemical compounds (Jacquemoud & Baret, 1990; Ustin *et al.*, 2009). pPC axis 3 (4.46 ± 0.43% of variation) appears to comprise mainly variation within the visible (VIS) wavelengths associated with both photosynthetic and accessory pigments (Curran, 1989; Jacquemoud & Baret, 1990; Ustin *et al.*, 2009). These traits underlying spectral variation for pPC axes 1 and 3 are under different selective pressures that may be associated with ecological strategies beyond mycorrhizal type. By contrast, pPC 2 represents traits that are under selection toward a single optimum regardless of mycorrhizal type. The traits underlying this spectral variation, representing 27.0 ± 1.8% of variation across the entire range of wavelengths, are likely conserved across the vascular plants, and these wavelengths may be strongly influenced by water content.

Lack of spectral differences between AM and EM species

Mycorrhizal associations have been linked to traits comprising the leaf economic spectrum, including leaf nutrients (Shi *et al.*, 2020), although the generality of this relationship has been called into question (Koele *et al.*, 2012). Leaf traits have in turn have been linked to leaf spectra (Serbin *et al.*, 2014; Ely *et al.*, 2019; Girard *et al.*, 2020; Kothari *et al.*, 2022). Therefore, we expected to observe differences in spectra between AM and EM species due to the indirect association between root traits and leaf spectra via leaf traits. Consistent with the findings of Cornelissen *et al.* (2001) and Hayes *et al.* (2014), we observed significant differences in leaf nutrients and spectra between AM and EM species when not statistically accounting for phylogeny. However, when we accounted for phylogeny, the relationship between spectra and mycorrhizal type was insignificant. This is consistent with the lack of statistical support we found for differences in leaf traits between AM and EM species when accounting for phylogeny, and with the findings of Koele *et al.* (2012), where independent contrasts failed to find support for a universal relationship between leaf nutrients and EM/non-EM species. Because of few independent origins of mycorrhizal types, we have a reduction in statistical power for finding support for the relationship between mycorrhizal type and spectra. Significant differences in leaf nutrients between AM and EM species were found in a study of thousands of woody plant species across a global distribution while accounting for phylogeny, including more independent origins of the EM mycorrhizal type within angiosperms which potentially increased their statistical power (Averill *et al.*, 2019). Therefore, our study does not provide evidence that there is no relationship between spectra and mycorrhizal type; rather, we lack evidence that these traits show correlated evolution.

The two modeling approaches used here (multivariate PCM and univariate models) complement each other and provide

different insights into the relationship between mycorrhizas and spectra. The univariate models examine the evolutionary patterns of spectra across the phylogeny with mycorrhizal type associated with possible alternative selective regimes. This approach does not remove the phylogenetic correlation but rather attempts to explain the variation in spectra across the phylogeny using mycorrhizal type. By contrast, PCM remove the variation attributed to phylogeny and attempt to explain the remaining spectral variation using mycorrhizal type. As a result, the PCM addresses spectral variation beyond phylogenetically correlated variation, while the univariate models address the phylogenetically correlated variation itself. The debate about attributing all variation correlated with phylogeny solely to phylogeny rather than to other associated forces is beyond the scope of this paper, but it is important to note that the variation that is associated with phylogeny is real variation. As illustrated in Fig. 2(a), there are visible differences between the mean spectra for AM and EM species (albeit with high variance), where AM species tend to have higher reflectance than EM species, in contrast with Sousa *et al.* (2021), who observed higher reflectance values in EM compared with AM tree species. However, it is important to account for evolutionary history when testing whether this variation is statistically associated with our predictor variables because of the impact of nonindependence on the likelihood of observing a significant relationship.

Other considerations and future directions

While the multivariate PCM implemented here have been used in other studies of datasets with higher numbers of traits than taxa, this study examines the most highly dimensional data to date. Spectra represent an aggregate of traits, with many different factors affecting this phenotype. With these highly dimensional data, it may be challenging to identify a single predictor that is significantly correlated with spectral variation from among the many interacting influences on spectra, even if the predictor has a direct influence on spectra. The complexity of mycorrhizal associations may influence this relationship. The EM type comprises multiple different kinds of ectomycorrhizal associations based on the fungal partner, which can impact the influence that the EM has on the host plant (Tedersoo, 2017). The impact of mycorrhizal association on the host can also depend on the mycorrhizal status (i.e. obligately or facultatively mycorrhizal; Brundrett, 2002; Hempel *et al.*, 2013) and environmental conditions (Näsholm *et al.*, 2013; Averill *et al.*, 2019). Therefore, the relationship between mycorrhizas and leaf spectra is much more complex than a binary AM vs EM designation and deserves continued in-depth analysis, including explicitly accounting for environmental impacts on the influence of mycorrhizal interactions on plant traits. While our dataset includes environmental variation, including intraspecific variation across nutrient gradients, our analyses did not directly examine how this variation could affect the covariance between mycorrhizal type and the traits underlying spectra.

Additionally, while PCM attempt to solve the problem of evolutionary correlation, the problem of singular evolutionary events remains (Uyeda *et al.*, 2018). Due to the coincidence of morphologically distinctive leaves and mycorrhizal type in conifers and angiosperms, these traits may appear to be correlated within this evolutionary context regardless of causative factors. Applying solutions to rare events like these, such as graphical model methods, may be a next step to examining the relationship between mycorrhizal type and leaf spectra (Uyeda *et al.*, 2018). Taxon sampling can also influence the ability of PCM analyses to find statistical support. Our study focused on temperate vascular plants of Canada, excluding a wide variety of tropical plants. In their study of global mycorrhizal associations, Averill *et al.* (2019) examined the relationship between leaf traits and mycorrhizal type with wider taxon sampling across woody vascular plants including tropical lineages and more independent origins of the EM type but excluding herbaceous plants. A truly global analysis may identify relationships between mycorrhizal type and leaf traits or spectra that have remained undetected.

Our findings highlight the importance of accounting for evolutionary history when investigating ecological questions such as the impact of mycorrhiza on spectral variation. However, while the ability of models to predict mycorrhizal type from leaf spectra may not be due to a direct influence of mycorrhizal type on leaf spectra, we show that belowground traits can be identified using leaf spectra, supporting the use of remotely sensed spectra to address pressing questions about the ecology and conservation of biodiversity.

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Competing interests

None declared.

Author contributions

JRJ, AB and EL developed the research question. AC compiled mycorrhizal data. RB-R, FB, ALC, AG, PWH, JP, AKS and SD-T collected the spectral and leaf trait data. EL, AB, NCC, MK and MV contributed to study design for spectral and trait data collection. JRJ conducted analyses and drafted the manuscript. All authors contributed to and reviewed the final manuscript.

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Data availability

The spectral and leaf trait data are available through the CABO data portal (https://data.caboscience.org/leaf). Data used in these analyses are archived on Zenodo (doi: 10.5281/zenodo.7683306). Scripts are available at https://github.com/jjantzen/Spectral_evolution.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Distribution of sampling locations across phylogeny.

Fig. S2 Number of sampling locations for each species colored by mycorrhizal type.

Fig. S3 Number of arbuscular mycorrhizal and ectomycorrhizal species at each sampling location.

Fig. S4 Distribution of 100 randomly sampled BEAST trees.

Fig. S5 Consensus tree with support values.

Fig. S6 Partial least squares discriminant analysis kappa plot for all taxa.

Fig. S7 Partial least squares discriminant analysis kappa plot for angiosperms only.

Fig. S8 Model parameter distributions for spectral intercept models.

Fig. S9 Traitgrams for phylogenetic principal component scores of axes 1–3.

Fig. S10 Model assessment plots for untransformed spectral data.

Fig. S11 Model assessment plots for transformed spectral data.

Fig. S12 Loadings for phylogenetic principal component axes 1–5.

Fig. S13 Univariate regressions for angiosperms only.

Fig. S14 Univariate modeling results with phylogenetic principal component scores and optima for angiosperms only.

Fig. S15 Traitgrams of phylogenetic principal component scores for angiosperms only.

Methods S1 Phylogenetic methods.

Methods S2 Angiosperm-only analyses.

Table S1 Overview of CABO projects contributing spectral data.

Table S2 Species names with sampling location.

Table S3 Sampling locations with list of species.

Table S4 Savitzky–Golay filter parameters by wavelength regions.

Table S5 Summary of sequences retrieved from GenBank using PyPHLAWD.

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Table S7 Calibration ages for BEAST analysis.

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Table S9 Overview of OUwie evolutionary models.

Table S10 Phylogenetic signal of predictor variables as measured by the *D* statistic and Delta.

Table S11 Multivariate intercept model parameters for best fitting models.

Table S12 Number of phylogenetic replicates where each evolutionary model was identified as best fit (highest AIC weights) for the top three phylogenetic principal component axes.

Table S13 Univariate modeling results for angiosperms only.

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