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Functional Traits in an Evolutionary Radiation: The Role of the Environment in the Diversification of Protea (L.)

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Functional Traits in an Evolutionary Radiation: The Role of the Environment in the Diversification of *Protea* (L.)

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Evolutionary radiations are responsible for much of the diversity on Earth, but the mechanisms leading to rapid lineage diversification and expansive morphological diversity are not always clear. Diversification may be the result of neutral processes, such as drift, or adaptive processes like adaptation to the abiotic or biotic environment. Flowering plants represent an extreme example of a radiation, and radiations at the tips of the angiosperm phylogeny are ideal places to examine the evolutionary relationships between plant traits and environmental factors. These relationships help us to understand the role of abiotic factors in driving the evolution of plant form. This dissertation uses the radiation of the flowering shrub genus *Protea*, a diverse group predominantly found in the Cape Floristic Region of South Africa, a biodiversity hotspot, to examine the relationships between functional traits (those with presumed consequences for survival) and the environments in which traits are found across different scales. Chapter 1 uses field-collected plant traits and databased climatic variables to compare trait-environment relationships between *Protea* and a parallel radiation in the genus *Pelargonium*. In these two distinct lineages, there is support for some associations in the same direction, while there is evidence for conflict in others. Chapter 2 provides a new phylogeny for *Protea* using a targeted-capture approach to sequence almost 500 nuclear genes. Species-level relationships are well-resolved, with differences between input gene trees and resultant species trees mostly due to a lack of information associated with short branch lengths. Chapter 3 uses the phylogeny from Chapter 2 to examine the joint evolutionary history of traits and environments in *Protea*, and finds that most associations are consistent across contemporary and evolutionary scales, with no strong evidence for either *in situ* adaptation or environmental filtering driving current patterns. Chapter 4 provides evidence for trait-environment relationships at the microgeographic scale in two closely-related species of *Protea* and tests these patterns in a controlled

greenhouse experiment on seedlings. Although there are detectable relationships at the microgeographic scale, they are not found in the greenhouse, implying that plasticity may be driving associations at the micro-scale.

Functional Traits in an Evolutionary Radiation: The Role of the Environment in the Diversification of *Protea* (L.)

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APPROVAL PAGE

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Functional Traits in an Evolutionary Radiation: The Role of the Environment in the Diversification of *Protea* (L.)

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To my parents, Judy and Tom Mitchell, for fostering my love of plants and nature, and encouraging me to choose and follow my own path. I dedicate this work to my father, Tom Mitchell (1954 – 2010).

Introduction

A central question in evolutionary biology is how the incredible assembly of species and morphological forms on Earth came to be. Understanding patterns in rates of speciation and phenotypic divergence, and the potential mechanisms driving these patterns are some of the keys to answering that question. Evolutionary radiations are ideal systems for approaching this puzzle, since these lineages rapidly diversified into a variety of new forms, and they are thought to make up much of Earth's diversity (Schluter 2001). Many factors can drive diversification, including both adaptive and non-adaptive processes. In some cases, natural selection may be the driving force, resulting in an adaptive radiation. In other cases, non-adaptive processes (such as genetic drift or chromosomal re-patterning) could be the primary mechanisms causing diversification (Kozak et al. 2006). In most radiations, both adaptive and non-adaptive processes have probably played a role.

To the extent that radiations are adaptive, the environment has is expected to have played a role in shaping patterns of morphological diversity. The classic Simpsonian model of radiation suggests that a lineage must first enter a new adaptive zone and can subsequently diversify into new forms to fill so-called "empty" niches (Simpson 1944). Recent examples support this idea. For example, in the radiation of *Anolis* lizards, there is a strong correlation between the perching habit and limb morphology that has been repeated on distinct islands (Losos 1990; Yoder et al. 2010). Perhaps the most famous adaptive radiation is the cichlid fishes in East African lakes, where some 2,000 species have evolved in just 10 million years. This radiation has been well studied, and it is hypothesized that habitat differentiation is the driving force behind diversification (Kocher 2004; Seehausen 2006).

Flowering plants (angiosperms) are an extreme radiation; Darwin called their rapid origin and diversification an "abominable mystery" (Friedman 2009). Within this larger radiation are many more recent radiations at lower taxonomic levels, many of which are associated with shifts in habitat or climate regime. For instance, the Hawaiian silversword alliance is a classic plant radiation, comprised of 28 species in three endemic genera, which displays a huge range of morphological and physiological

diversity (Robichaux 1990). Similarly, the Hawaiian lobeliads, which have 126 species in 6 genera, show patterns of radiation driven by both habitat and pollinator diversity (Givnish et al. 2009).

If radiations are characterized by both speciation and morphological diversification, we might expect fine-tuned associations between plant functional traits, those that indirectly impact fitness (Violle et al. 2007), and specific environmental variables. These traits are often continuous, and specific traits are expected to evolve in predictable ways in response to different environmental or climatic conditions which are also continua. The much-cited "worldwide leaf economics spectrum" (Wright et al. 2004; Wright et al. 2005) and corresponding wood (Chave et al. 2009) and whole-plant (Poorter et al. 2014; Reich and Cornelissen 2014) spectra have identified strategies that are repeatedly selected for survival under different environmental conditions and stresses. However, the extent to which these global patterns hold up at regional or more local scales has been questioned (Wright and Sutton-Grier 2012; Kang et al. 2014; Laforest-Lapointe et al. 2014; Mason and Donovan 2015), and the role of the environment in actually driving the evolution of these traits is difficult to assess (but see Evans et al. 2009; Kozak and Wiens 2010). Additionally, the causes of these trait-environment patterns may be different at different scales (Messier et al. 2010; Messier et al. 2016). Patterns across species or populations may be driven by local adaptation, but phenotypic plasticity or environmental filtering could also play roles at local, regional scales, or biome-level scales (Diaz et al. 1998; Ackerly and Cornwell 2007; Bello et al. 2013)

The Cape Floristic Region (CFR) of South Africa provides an ideal opportunity to investigate plant radiations and the role of the environment in shaping functional traits. The CFR, located in the southwestern portion of South Africa, is a biodiversity hotspot characterized by incredible species diversity (9,000 species of plants) and high levels of endemism (70%) (Goldblatt and Manning 2002; Linder and Hardy 2004). Much of the habitat is threatened, making it an area of conservation concern (Myers et al. 2000). In addition, much of this diversity is accounted for by radiations in just 33 lineages (Linder 2003). There are many hypotheses about the origins of this diversity, including extreme climatic shifts during the late Miocene, such as the upwelling of the Benguela current, resulting in aridification and a shift towards a Mediterranean climate dominated by cool, wet winters and dry summers (Linder

2003; Linder 2005). This region is also topographically complex, edaphically heterogeneous, dominated by fire regimes, and composed of multiple climatic gradients in temperature, and amount and seasonality of rainfall, all of which have also been proposed as drivers of diversification (Linder 2003; Dupont et al. 2011; Schnitzler et al. 2012). Some combination of these factors has resulted in high beta diversity (turnover in species), often associated with multiple biome types dominated by different assemblages of vegetation (Cowling 1990). This region is not only extremely environmentally complex, but is also predicted to undergo drastic changes in many key gradients under current climate change models, making the past evolutionary history relevant to its future (Wilson et al. 2015).

The genus *Protea* L. is an iconic lineage in the fynbos biome, and is a symbolic plant group in South African culture. This clade, within the family Proteaceae, is species rich (~112 taxa), and has its center of diversity in the CFR, where 60% of its species occur (Goldblatt and Manning 2000). *Protea* displays a wide range of growth forms, from ground-dwelling individuals, to shrubs and trees, as well as a variety of leaf shapes and sizes often related to leaf function (Rebelo 2001). The distribution and occurrence of *Protea* species has been well-documented by the Protea Atlas Project, which facilitates sampling of individuals in the field as well as climatic niche modeling (Protea Atlas Project, available at www.proteaaltas.org.za). *Protea* is thus an ideal group for investigating many questions, ranging from physiology to demographic trends, diversification rates, and biogeographical hypotheses (Valente et al. 2010; Yates et al. 2010; Merow et al. 2014). A smaller clade within the genus, the white protea clade, has been used as a tool to investigate population genetic questions and the roles of adaptation and phenotypic plasticity in influencing plant traits and performance (Latimer et al. 2009; Prunier and Holsinger 2010; Carlson et al. 2011; Prunier et al. 2012).

In Chapter 1, I ask whether there are detectable trait-environment relationships in two distinct plant genera within a single biome of the CFR. If unrelated plant lineages evolve in similar ways to the same environmental pressures, then traits and environment should correlate in the same ways. I used field-collected plant trait data and fine-scale environmental database measures to estimate trait-environment associations in both *Protea* and a distantly related genus with a similar geographic

distribution, *Pelargonium*, using Bayesian multivariate multiple-response models incorporating phylogeny. I compared twenty-four pairwise trait-environment associations between the two genera to assess the extent to which these groups have evolved traits in similar or different ways. I found that although some associations were in the same direction, they were not always consistent across genera or with global trends, indicating that lineage-specific attributes were also important. *This work has been published in the American Naturalist (Mitchell et al. 2015).*

In Chapter 2, I build a well-resolved phylogeny for *Protea* using a targeted-capture approach. It is difficult to estimate evolutionary relationships in a radiation where diversification has occurred rapidly, resulting in short branch lengths and a lack of phylogenetic information. I used anchored phylogenomics to capture and sequence DNA for almost 500 nuclear genetic markers, built individual gene trees for each locus, and used four different species-tree building approaches to estimate phylogenies for the genus. I compared gene-trees with species-trees and used a network-based approach to understand whether branches low phylogenetic support are associated with incomplete lineage sorting (ILS) or hybridization. I found that species tree topologies were largely consistent, though differences between species and gene trees are likely due to few informative sites and a lack of information. *This work has been published in the American Journal of Botany (Mitchell et al. 2017).*

In Chapter 3, I ask whether plant functional traits in *Protea* have co-evolved with the environments in which they are found. Contemporary associations between traits and environment are indicative of adaptive radiation, but phylogenetic analyses are needed to further assess the role of adaptation. I use phylogenetic comparative analyses on field-collected trait data to find evidence for both contemporary and evolutionary patterns of trait-environment correlations. I ask if these associations are due to environmental filtering or *in situ* adaptation by analyzing evolutionary models and the timing of divergence of both traits and environment. To assess the consequences of uncertainty in evolutionary relationships and intraspecific trait variation, I incorporate samples from bootstrap replicates of phylogenies, Bayesian modeling posteriors, and maximum-entropy models into my analyses.

Finally, in Chapter 4 I ask if there are detectable trait-environment associations at the local scale, and explore the causes of these associations. Global relationships are not always upheld at finer scales, and the causes of trait-environment correlations are likely different across ecological scales. I focus on a small geographic scale to detect trait-microenvironment associations and use a controlled greenhouse stress experiment to understand whether environmental filtering or phenotypic plasticity is driving relationships in the field.

This dissertation investigates the role of the environment in shaping the evolution of species and morphological diversity of plants, using the genus *Protea* as an exemplary system. This work increases our understanding of mechanisms underlying rapid evolution in an important plant lineage and it may help to predict how climate change and new environments will affect diverse plant groups. My research takes a multi-level approach, from contemporary observable patterns, to phylogenetic history across a group, and finally experimental evidence for mechanisms underlying traits indirectly responsible for plant survival and fitness.

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Chapter 1:

Functional traits in parallel evolutionary radiations and trait-environment associations in the Cape Floristic Region of South Africa

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January 11, 2017

Nora Mitchell
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Re: your 11 January letter

Dear Ms. Mitchell;

The University of Chicago Press is happy to grant you permission to include your recent *American Naturalist* contribution, "Functional Traits in Parallel Evolutionary Radiations and Trait-Environment Associations in the Cape Floristic Region of South Africa" co-written with Timothy E. Moore, Hayley Kilroy Mollmann, Jane E. Carlson, Kerri Mocko, Hugo Martinez-Cabrera, Christopher Adams, John A. Silander Jr., Cynthia S. Jones, Carl D. Schlichting, and Kent E. Holsinger [*The American Naturalist* Apr 2015, Volume 185, Issue 4, pp. 525 – 537] in your dissertation.

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Functional Traits in Parallel Evolutionary Radiations and Trait-Environment Associations in the Cape Floristic Region of South Africa

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abstract: Evolutionary radiations with extreme levels of diversity present a unique opportunity to study the role of the environment in plant evolution. If environmental adaptation played an important role in such radiations, we expect to find associations between functional traits and key climatic variables. Similar trait-environment associations across clades may reflect common responses, while contradictory associations may suggest lineage-specific adaptations. Here, we explore trait-environment relationships in two evolutionary radiations in the fynbos biome of the highly biodiverse Cape Floristic Region (CFR) of South Africa. *Protea* and *Pelargonium* are morphologically and evolutionarily diverse genera that typify the CFR yet are substantially different in growth form and morphology. Our analytical approach employs a Bayesian multiple-response generalized linear mixed-effects model, taking into account covariation among traits and controlling for phylogenetic relationships. Of the pairwise trait-environment associations tested, 6 out of 24 were in the same direction and 2 out of 24 were in opposite directions, with the latter apparently reflecting alternative life-history strategies. These findings demonstrate that trait diversity within two plant lineages may reflect both parallel and idiosyncratic responses to the environment, rather than all taxa conforming to a global-scale pattern. Such insights are essential for understanding how trait-environment associations arise and how they influence species diversification.

Keywords: functional traits, multiple-response model, *Protea*, *Pelargonium*, plant strategies, phylogenetic mixed model.

Introduction

The concept of functional traits has long been used as a tool for understanding how plants adapt to their environment and which environmental factors influence their distribution and abundance (Warming 1895; Schimper 1898; Tilman 1984; Cavender-Bares et al. 2004). The term "functional trait" is typically applied to the "morphological, chemical, physiological and phenological attributes of plants that interact with surrounding biotic and abiotic factors" (Drenovsky et al. 2012, p. 142) or to "any trait which impacts fitness indirectly via its effects on growth, reproduction, and survival" (Violle et al. 2007, table 3, p. 889). Ecological studies of functional traits have tended to use global data sets spanning many biomes and distantly related species (Reich et al. 1999; Wright et al. 2002; Chave et al. 2009; Meng et al. 2009; Sandel et al. 2010; Emery et al. 2011; Dwyer et al. 2014; Moles et al. 2014). For example, the widely cited worldwide analysis of the leaf economic spectrum (Wright et al. 2004) used the GLOPNET data set comprising 2,548 species and 175 globally distributed sites and concluded that the association of leaf traits and trait relationships with climate is weak. In contrast, evolutionary studies have focused on determining whether functional trait differences among populations or closely related species are adaptive and have often identified strong relationships to environmental conditions when they are (Clausen et al. 1940; Linhart and Grant 1996; Ellis and Weis 2006).

When strong associations occur between functional traits and the environments species occupy, shifts in these traits within clades are often attributed to evolutionary radiations into novel environments (Klak et al. 2004). Such radiations may be responsible for most of the world's biodiversity (Schluter 2001). Simpson's (1944) original concept of an adaptive zone centered on the idea that lineages entering an empty adaptive zone would rapidly diversify to fill

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empty niches and that such diversification would be primarily adaptive. To the extent that Simpson's model and more recent versions of it (Gavrilets and Vose 2005; Agrawal et al. 2009; Gavrilets and Losos 2009) hold, evolutionary radiations should lead to strong associations between species functional traits and the environments they occupy. While several nonadaptive processes could lead to trait-environment associations, a failure to detect such associations would suggest that adaptation in the measured traits did not play a large role in the evolutionary radiation.

The Cape Floristic Region (CFR) of South Africa boasts a unique flora dominated by endemic plant species, approximately 70% (Goldblatt and Manning 2002; Linder and Hardy 2004), and much of the diversity is due to evolutionary radiations in just a few clades (Schnitzler et al. 2011). Many of the radiations are presumed to be relatively recent (Linder and Hardy 2004), but the relative importance of adaptive and nonadaptive processes in driving these radiations remains controversial (Linder and Vlok 1991; Linder 2003; Verboom et al. 2004; van der Niet and Johnson 2009; Johnson 2010; Britton et al. 2014). Nonetheless, strong climate gradients may be associated with functional divergence and even speciation in the CFR (Richardson et al. 2001; Verboom et al. 2003; McKenzie and Barker 2008), and a number of studies have identified trait-environment associations in different plant groups occurring in the region (Thuiller et al. 2004; Nicotra et al. 2008; Yates et al. 2010; Prunier et al. 2012; Jones et al. 2013). Carlson et al. (2011) showed that among-population differences within a small clade in the genus *Protea* were associated with major climatic axes in the CFR. Common-garden experiments suggested that these relationships involve functional traits under genetic control, and the association of trait differences with survival suggested that they were adaptive (Carlson et al. 2011; Carlson and Holsinger 2012). Another recent study showed that functional groups within the CFR may respond to their environments differently, with different growth forms showing significantly different responses under the same experimental setting (West et al. 2012). In this study we investigate whether two genera that have their centers of diversity in and are found throughout the CFR, *Protea* L. and *Pelargonium* L'Her ex Aiton, have detectable trait-environment associations and whether these responses are congruent across lineages. The presence of these associations might suggest a role for the environment in promoting evolutionary divergence, while consistent associations across genera would suggest that the environment shapes functional traits among populations and species in similar manners in spite of their very different growth forms and life histories.

Previous studies of trait-environment associations (Wright et al. 2004; Yates et al. 2010) have focused primarily on relationships between one trait and one environmental variable at a time (but see Cornwell and Ackerly 2009; Pollock et al. 2012). The environments a plant experiences, however, vary on several axes, and plants experience their environment as an integrated whole. There are, therefore, potentially a wide range of trait combinations that may be adaptive in a given environment (Marks and Lechowicz 2006). In this study we examine the joint association of several environmental variables with a multivariate vector of plant functional traits. We present an integrated assessment of associations between four different traits and six environmental covariates to address the following questions: (1) Are there detectable associations between functional traits and climatic features in these evolutionary radiations consistent with environmental adaptation? (2) Can we detect similarities in pairwise trait-environment associations between *Protea* and *Pelargonium*, consistent with similar adaptive responses to environmental gradients?

Material and Methods

Taxon Sampling

We focused on two genera that typify the CFR yet are strikingly different in numerous ways. The genus *Protea* (~112 species) is at its most diverse in the CFR, with approximately 60% of species occurring within the region (>90% of these are CFR endemics), although representatives of the genus extend as far as 15°N in Africa (Rourke 1980; Rebelo 2001; Valente et al. 2010). The genus displays great morphological variation, ranging from low-growing shrublets with belowground or sprawling stems to small trees. Leaf sizes and shapes also vary markedly among species (fig. 1), and these differences are functionally significant (Yates et al. 2010). Similarly, the genus *Pelargonium* contains approximately 280 species, found primarily in southern Africa, and with 70% of those species endemic to the CFR, it is the third-largest genus in the region (Goldblatt and Manning 2000). However, in contrast to *Protea*, *Pelargonium* includes annuals, stem succulents, geophytes, and small shrubs, both evergreen and deciduous (Goldblatt and Manning 2000), and displays extraordinary leaf variation (fig. 1; Jones et al. 2009), ranging from large entire leaves to small finely dissected or needlelike leaves. Although both genera occupy broadly similar environments and representatives of both are found together at many sites within the CFR, they differ markedly in life history and phenology. In particular, *Protea* leaves are sclerophyllous and persist through summer drought periods (Coetzee and Littlejohn 2007), while *Pelargonium* leaves are often drought-deciduous.

We collected data from 45 *Protea* and 52 *Pelargonium* species across the CFR of South Africa in 2011, 2012, and 2013, with many species sampled at several sites and 4–8

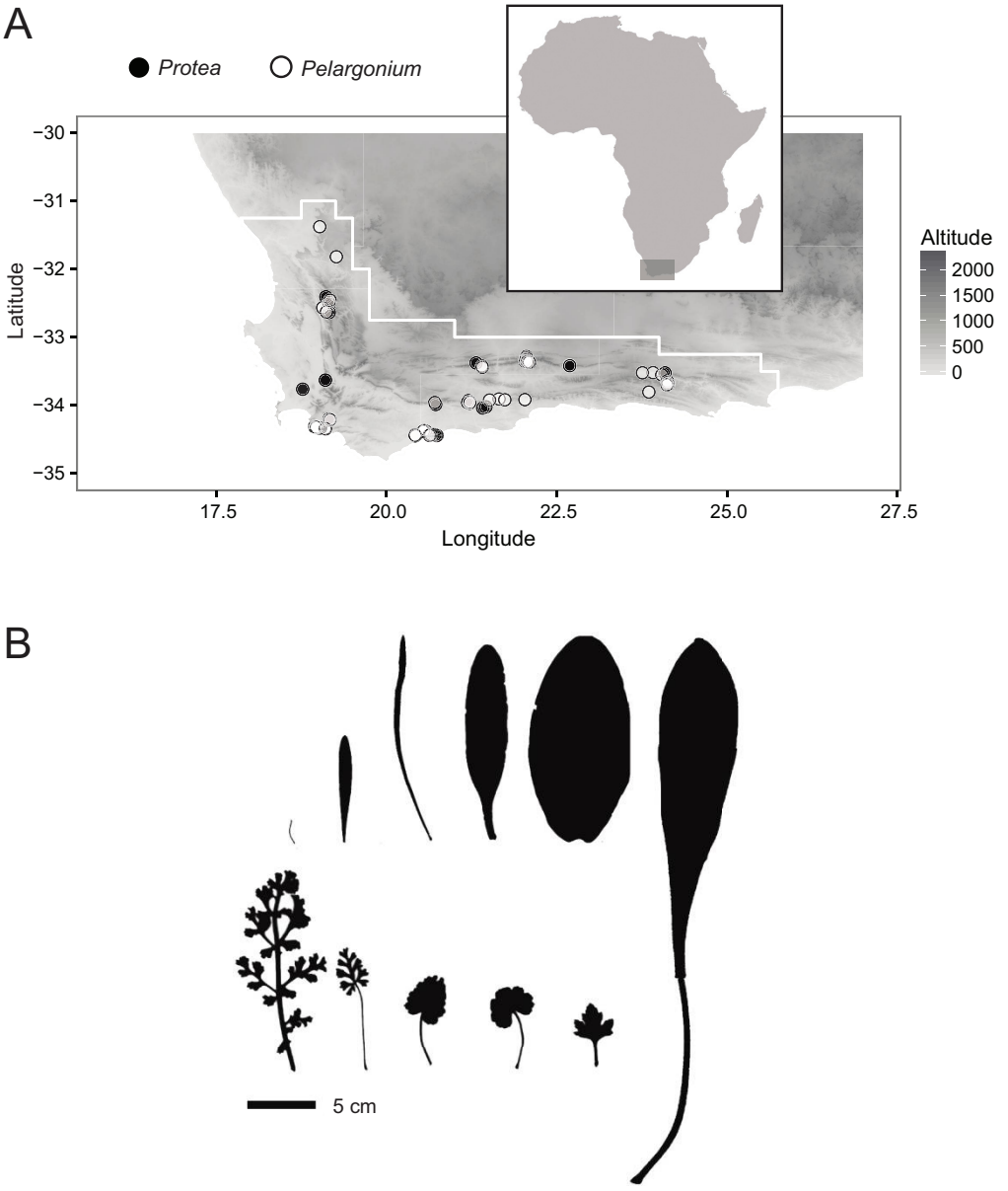

Figure 1: Field sampling and leaf diversity in *Protea* and *Pelargonium*. *A*, Sampling localities for *Protea* (black) and *Pelargonium* (white); the outline indicates the Cape Floristic Region GIS layer from which environmental variable values were extracted (Wilson and Silander 2014). *B*, Examples of leaf diversity in *Protea* (*top row*) and *Pelargonium* (*bottom row*).

adult individuals measured in each population. Collection sites for *Protea* were identified from the Protea Atlas Database (http://www.proteaatlas.org.za/index.htm, Rebelo). Collection sites for *Pelargonium* were identified from historical collection records found in the PRECIS (Morris and Glen 1978) and Acocks (Rutherford et al. 2003) databases. Of the species in the CFR, our samples represent roughly two-thirds of *Protea* and roughly one-third of *Pelargonium*. In both genera, our sampling spans most of the phylogenetic diversity and includes representatives of all major clades in the CFR. Our sample consists of 165 distinct site-species combinations, 74 in *Protea* and 91 in *Pelargo-*

nium (see fig. 1 for map of sampling localities), and 1,223 individual plants over the 3-year period. Populations per species ranged from 1 to 7 in *Protea*, with 17 species represented by multiple populations, and from 1 to 5 in *Pelargonium*, with 21 species represented by multiple populations. A list of all species and population locations sampled has been deposited in the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.sc286 (Mitchell 2014). Voucher specimens were deposited at the Compton Herbarium (PRE, South African National Biodiversity Institute) and the George Safford Torrey Herbarium (CONN, University of Connecticut).

Trait Measurements

Teams measured a suite of physical traits on 4–8 individuals per population on plants and leaves taken from the field, including canopy area (estimated as the area of an ellipse defined by the longest diameter and the orthogonal diameter), as well as the leaf area, fresh and dry weight of leaves, leaf length, and leaf functional width. Values used are for individual plants and leaves, not population or species means, which can obscure variation. Leaf traits include functional width, which is a measurement related to the leaf boundary layer (Nobel 1975) and is defined as the diameter of the largest circle that can be fitted in a leaf. Thus, functional width does not include extensions of the leaf blade associated with lobes or dissection, which is a key distinction between the entire leaves in *Protea* and the lobed or highly dissected leaves in *Pelargonium* (fig. 1). Using the primary leaf measurements, we calculated the following derived measures (Peek 1970; Vendramini et al. 2002; Shipley et al. 2006; Poorter et al. 2009; Osnas et al. 2013): leaf mass per area (LMA), leaf fresh water content (FWC; [fresh weight − dry weight]/dry weight), and leaf length–to–leaf width ratio (LWR). See table 1 for a description of traits used in this analysis.

We use canopy area as a proxy for overall plant size or biomass to reflect the total amount of resources put into a single individual (neglecting belowground biomass). It can also be used as an index of plant performance (Violle et al. 2007). LMA is an indicator of leaf toughness or sclerophylly and is often related to leaf longevity and investment. It is a central component of the worldwide leaf economics spectrum (Wright et al. 2002, 2004). FWC is a measure of water content by mass. Although LMA and FWC are negatively correlated (fig. A1; figs. A1, A2 available online), they capture different functional aspects of plant morphology. In the taxa included in this study, higher LMA is associated with tougher, more sclerophyllous leaves (Lamont et al. 2013), while higher FWC represents fleshier, more succulent leaves (Jones et al. 2013). Finally, LWR may be associated with temperature regulation and nutrient uptake. Thinner, narrower leaves, or leaves that are highly dissected (both resulting in high LWR) are expected to facilitate cooling by increasing transpiration rates via a thinner boundary layer (Yates et al. 2010).

Environment

We focus on environmental variables that capture the major gradients in climate across the CFR, including a strong east-west rainfall seasonality gradient and a north-south aridity gradient, as well as variables that allow us to compare our analyses with trait-environment relationships in the literature. We performed our sampling at a fine population-level scale, so we used a climatic database with daily precipitation and temperature measurements interpolated across the landscape at 1-min (1.55 km × 1.85 km) spatial resolution (Wilson and Silander 2014). Extreme

Table 1: Plant traits and environmental variables used in multiple-response model

Variable	Description	Units
Traits/responses:		
LMA	Leaf mass per area	g cm^{-2}
LWR	Leaf length–to–leaf width ratio	NA
FWC	Leaf fresh water content	g g_{dw}^{-1}
AREA	Area of ellipse defined by longest diameter and orthogonal diameter of the canopy	cm^2
Environments/covariates:		
CDD	Consecutive drought days	days
ELEV	Elevation	m
INSO	Insolation	W m^{-2}
MAP	Mean annual precipitation	mm $year^{-1}$
MAT	Mean annual temperature	°C
RATIO	Winter-to-summer rainfall ratio	NA

Note: NA = not applicable.

events that influence plant responses (Gutschick and Bassiri-Rad 2003) may be imperfectly reflected in monthly data sets such as WorldClim (Hijmans et al. 2005). Environmental measurements for all sites included in our sample were downloaded from the database described by Wilson and Silander (2014), which includes both daily measurements and summary metrics derived from those daily values for 20 years (1990–2009). We extracted the median values of summary variables for every year in the data set and then calculated 20-year averages for each of them. We calculated mean annual temperature (MAT) by averaging the minimum and maximum temperatures for each day to calculate mean daily temperatures, computing the annual means, and then extracting the 20-year average. From the metrics summarized by Wilson and Silander (2014), we chose mean annual precipitation (MAP) and consecutive drought days (CDD) to reflect the aridity gradient. To reflect the marked gradient in rainfall seasonality in this region dominated by a Mediterranean climate and winter rain but also extending into aseasonal rainfall toward the east, we calculated a derived variable "ratio" as the amount of winter rainfall (April–September) minus the amount of summer rainfall (October–March) divided by the total yearly rainfall. To account for the topographical complexity within 1-min climate grid cells of Wilson and Silander (2014), we extracted elevation points from the ASTER Global Digital Elevation Map (GDEM; NASA Land Processes Distributed Active Archive Center; 30-m grid). We also extracted values for insolation from the ASTER GDEM, using the ArcGIS Spatial Analyst extension (ESRI 2014). To estimate insolation for each sample point, we transformed the DEM into a tiled mosaic of 100 × 100-km pieces and projected the DEM and GPS of points onto the projected coordinate system WGS 1984 UTMZone 34S. We used a time interval of a full year (2011, since most measurements were taken this year), with monthly intervals, with a sky size/resolution of 200, and with the remaining parameters kept at default. See table 1 for a description of environmental variables used in this study and figure A2 for correlations among environmental variables.

Phylogeny

Our statistical model requires a coancestry matrix describing the fraction of shared ancestry for every pair of species included in the analysis. To calculate the matrix for *Protea*, we downloaded the sequence data used in Valente et al. (2010) from TreeBase. We obtained a phylogenetic tree with branch lengths assuming exponential growth under a GTR model from BEAST running on the University of Oslo Bioportal. For *Pelargonium*, we used the topology from a 50% majority-rule tree by F. T. Bakker, E. M. Marais, R. Prunier, and A. S. J. van Proosdij (unpublished manuscript), based on rDNA ITS, cpDNA trnL-F, their indels, and mtDNA nad4 b/c exon sequences for 220 *Pelargonium* accessions, following Bakker et al. (2004). Bayesian sampling of trees and branch lengths was performed using Markov chain Monte Carlo (MCMC) analysis in MrBayes 3.2 with each chain running for 250 million generations on the XSEDE supercomputer at CIPRES Science Gateway platform (http://www.phylo.org). We added taxa sampled for traits that were not included in the phylogeny to the base of the clade containing the majority of the species from that section (see sections in Bakker et al. 2004). Species were added using the add.tip function in the R package ape v.3.0-8. Trees for both *Protea* and *Pelargonium* were ultrametricized using the compute.brlen function in ape v.3.0-8 with the "Grafen" method, power = 1. Sequence data and trees used are available in the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.sc286 (Mitchell 2014).

Analysis

Although our primary interest is to understand how our focal traits respond to focal environmental covariates, our analysis also explicitly addresses the influence of ancestry and intertrait correlations on these relationships. We take this approach because closely related species may share similar traits because of their close phylogenetic relationship rather than because of similarity in the environments they inhabit. Similarly, a significant relationship between a given trait and a given environmental covariate may be indirectly driven by covariation between two traits. Thus, rather than performing four separate multiple regressions investigating the relationship of each trait to the same suite of environmental covariates, we constructed a multiresponse multiple regression in which the suite of traits is regarded as a single vector-valued response. This approach also has the advantage of allowing us to incorporate covariation among the traits both at the level of variation among individuals within populations and at the level of trait evolution across the phylogeny. Because *Protea* and *Pelargonium* differ so greatly from each other, we analyzed the data from each genus separately and compared the results using posterior comparisons of the resulting regression coefficients (Holsinger and Wallace 2004).

We constructed the statistical model as follows. Our data consist of K trait measurements on I individuals at J locations. We have measurements of E environmental variables at each location, and we collected data from S species. The term $y_{ijk}^{(l)}$ is the observation of trait k in individual i at location j. The superscript (l) indexes the species to which this individual belongs. Each observation is modeled as a linear relationship with environmental covariates, a random phylogenetic effect (reflecting species relation-

Additional online supplementary material for Chapter 1 may be found at:

Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.sc286

ships), and a random individual residual error effect (reflecting variation among individuals within a species at a particular sample site),

$$y_{ijk}^{(l)} = \beta_k^T x_j + \phi_k^{(l)} + \epsilon_{ijk}^{(l)},$$
$$y_{ij.}^{(l)} = \beta^{(T)} x_j + \phi^{(l)} + \epsilon_{ij}^{(l)},$$
$$\epsilon_{ij}^{(l)} \sim N\left(0, \sum^{(e)}\right),$$

where $y_{ij.}^{(l)}$ is the vector of trait values, x_j is the vector of environmental covariates, β^T is the corresponding matrix of regression coefficients, $\phi^{(l)}$ is the vector of phylogenetic random effects, and $\epsilon_{ij}^{(l)}$ is the vector of residual errors. As indicated above, we model $\epsilon_{ij}^{(l)}$ as multivariate normal, with $\sum^{(e)}$ as a block diagonal. The blocks of $\sum^{(e)}$ represent the covariance of traits within individuals. For $\phi^{(l)}$ we follow the approach in Lynch (1991), generalizing it to account for coevolution of multiple traits (see also Hadfield and Nakagawa 2010). Specifically,

$$\phi \sim N\left(0, \sum^{(p)}\right),$$
$$\sum^{(p)} = G \otimes \Delta,$$

where G is a coancestry matrix in which G_{ij} is the fraction of phylogenetic history shared by individuals i and j (1 for conspecifics, 0 for individuals belonging to species on branches separated by the root of the phylogeny) and Δ is the matrix describing the covariance of traits over the evolutionary history of the group.

We calculated G from the rate-smoothed phylogenies using vcv() from ape v3.0-8 in R (R Core Development Team 2013). We standardized all response variables and covariates to a mean of 0 and standard deviation of 1 prior to the analysis in order to compare regression coefficients between variables on the same scale and specified vague normal priors for the regression coefficients (mean = 0, variance = 10). We constructed priors on the covariance matrices from priors on individual variances and on pairwise correlations. Specifically, we specified independent $\gamma(1, 1)$ priors on each standard deviation and independent $\beta(6, 6)$ priors on correlation coefficients (transformed to lie in $[-0.5, 0.5]$). We implemented the model in JAGS v3.3.0 (Plummer 2003) and present results based on MCMC simulations of five chains with a burn-in of 5,000 iterations followed by a sample of 20,000 iterations (thinned to every twenty-fifth iteration), for an overall posterior sample of 4,000 points. Gelman and Rubin's (1992) Rhat was less than 1.01 for all parameters, indicating satisfactory convergence.

Because we are looking for broad patterns in the directionality of trait-environment relationships, we used a modified posterior comparison (Holsinger and Wallace 2004) to determine whether associations in *Protea* and *Pelargonium* are in the same or different directions. Specifically, for each sample from the posterior we determined whether regression coefficients (1) were both negative, (2) were both positive, or (3) were of opposite sign (in conflict). We report the posterior probability of each outcome as the proportion of MCMC samples falling into that category. The complete set of data and code for this work are deposited in the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.sc286 (Mitchell et al. 2014).

Results

Are There Detectable Associations between Functional Traits and Environmental Features in Protea *and* Pelargonium *Consistent with Environmental Adaptation?*

We detected a variety of trait-environment associations in both genera (table 2), which we analyzed as individual responses that take into account covarying relationships in both predictor and response variables. Across both genera, we detected significant relationships for 16 of the 24 possible associations in at least one genus. Only for FWC in *Protea* did we fail to detect any environmental associations. Of the 24 possible associations in each genus, we detected 9 relationships in *Protea* and 13 in *Pelargonium* such that in all we detected trait-environment associations in just under half (22 out of 48) of the cases we examined.

In *Protea* more sclerophyllous leaves (higher values of LMA) are associated with lower levels of MAP, higher elevations, and more concentrated winter rainfall (higher values of ratio; table 2). Narrower leaves (higher values of LWR) are associated with higher MAP, and narrow leaves are also associated with less concentrated winter rainfall. In short, sclerophyllous, broad leaves are associated with low levels of precipitation concentrated in the winter. In addition, larger canopy areas are associated with lower-elevation sites having fewer CDDs, lower temperatures, and lower rainfall.

In *Pelargonium*, more sclerophyllous leaves are associated with lower levels of rainfall and low temperatures but higher values of insolation. Fleshier leaves are associated with higher levels of rainfall, higher temperatures, and lower insolation. In short, sclerophyllous leaves containing little water are associated with low levels of mean annual rainfall and low temperatures. Narrower leaves are associated with lower MATs and fewer CDDs. In addition, larger canopy areas are associated with lower temperatures, lower elevations, more drought, and more concentrated winter rainfall.

Table 2: Regression coefficients for trait-environment relationships in *Protea* and *Pelargonium*

Trait	Environment	*Protea* coefficient (95% CI)	*Pelargonium* coefficient (95% CI)	Negative	Conflict	Positive
AREA	CDD	−.236 (−.387, −.087)	.346 (.200, .487)	.000	.999[a]	.001
AREA	ELEV	−.279 (−.544, −.022)	−.190 (−.379, −.013)	.966[a]	.034	.000
AREA	INSO	.014 (−.134, .156)	−.096 (−.204, .009)	.406	.572	.022
AREA	MAP	−.263 (−.471, −.052)	−.062 (−.231, .101)	.769	.230	.001
AREA	MAT	−.503 (−.795, −.218)	−.254 (−.383, −.125)	1.000[a]	.000	.000
AREA	RATIO	−.069 (−.274, .127)	.147 (.011, .287)	.014	.733	.253
FWC	CDD	.090 (−.025, .209)	−.020 (−.161, .124)	.040	.598	.362
FWC	ELEV	.072 (−.140, .286)	−.035 (−.224, .151)	.164	.575	.261
FWC	INSO	.009 (−.113, .126)	−.119 (−.225, −.014)	.424	.570	.005
FWC	MAP	.161 (−.004, .330)	.203 (.039, .372)	.000	.035	.965[a]
FWC	MAT	.210 (−.002, .422)	.258 (.127, .382)	.000	.026	.974[a]
FWC	RATIO	−.142 (−.299, .013)	−.073 (−.212, .07)	.809[b]	.187	.004
LMA	CDD	.087 (−.018, .198)	−.046 (−.180, .085)	.043	.715	.242
LMA	ELEV	−.214 (−.424, −.005)	.000 (−.175, .172)	.495	.493	.012
LMA	INSO	−.053 (−.163, .056)	.166 (.068, .263)	.001	.825[b]	.175
LMA	MAP	−.427 (−.587, −.265)	−.490 (−.645, −.335)	1.000[a]	.000	.000
LMA	MAT	.135 (−.078, .340)	−.376 (−.495, −.254)	.102	.898[b]	.000
LMA	RATIO	.457 (.306, .604)	.251 (.124, .382)	.000	.000	1.000[a]
LWR	CDD	−.035 (−.121, .056)	−.099 (−.175, −.020)	.787	.212	.001
LWR	ELEV	−.007 (−.185, .167)	−.027 (−.130, .074)	.376	.481	.142
LWR	INSO	.025 (−.071, .115)	.023 (−.031, .079)	.067	.369	.565
LWR	MAP	.172 (.042, .304)	−.091 (−.179, −.001)	.004	.973[a]	.024
LWR	MAT	−.051 (−.223, .121)	−.054 (−.123, .014)	.677	.306	.017
LWR	RATIO	−.209 (−.333, −.087)	.045 (−.030, .121)	.131	.869[b]	.000

Note: Values indicate the mean and 95% credible interval (CI) from the posterior distribution of our analysis. We also resampled from the posteriors to compare relationships between *Protea* and *Pelargonium* (final three columns). Negative is the posterior probability that both are negative, conflict is the posterior probability that they conflict, and positive is the posterior probability that both are positive. See table 1 for additional abbreviations of trait and environment.

[a] >95% posterior probability.

[b] Additional >80% posterior probability.

Are Pairwise Trait-Environment Associations Detected within Protea *Similar to Those Detected within* Pelargonium*?*

If we use 95% posterior probability as the criterion for good evidence of whether the trait-environment associations are in the same or opposite directions, we find only two cases where we detected strong evidence that the direction, that is, the sign, of trait-environment relationships differed (table 2). Specifically, large canopy areas are associated with short droughts in *Protea* and with long droughts in *Pelargonium*. Narrower leaves (higher LWR) are associated with increased annual precipitation in *Protea* but with less precipitation in *Pelargonium*. If we relaxed our criteria to 80%, we found an additional three cases of conflict. In *Protea* we detected a negative relationship between ratio and LWR, and there is little evidence of a relationship in *Pelargonium*. In *Pelargonium*, we found a positive relationship between insolation and LMA and a negative relationship between MAT and LMA, while the evidence for these relationships in *Protea* is equivocal.

We detected strong evidence (≥95% posterior probability) for six relationships in the same direction in both genera, three negative and three positive (table 2). For negative relationships, higher values of LMA are associated with lower levels of MAP while larger canopy areas are associated with lower MATs and lower elevations (table 2; fig. 2). The three positive relationships associated higher FWC with increased rainfall and annual temperatures, while LMA also increased with temperature (table 2; fig. 2). Relaxing the cutoff to 80% added only one additional negative relationship shared across both genera: FWC has a tendency to decrease with increased rainfall seasonality, but neither of these relationships is significant based on their 95% credible intervals (table 2).

In short, of the 24 trait-environment relationships that we observed in both genera, we have strong evidence for only two of the relationships differing in direction between *Protea* and *Pelargonium*. In addition, we have convincing evidence that 6 of the 24 were in the same direction. Even though few cases of conflict have strong support, conflict has the highest posterior probability in 12 out of 24 cases. Thus, *Protea* and *Pelargonium* could have different relationships in many cases, but the evidence for these differences is relatively weak.

Discussion

Trait-Environment Relationships in an Evolutionary Radiation

Evolutionary radiations are of extreme interest to the study of diversification and the origins of biodiversity and serve as ideal situations for assessing the possible role of the environment in promoting morphological shifts. If a radiation is not only evolutionary but also adaptive, we expect to detect associations between phenotype and environment as lineages morphologically differentiate to fill space within an adaptive zone (Schluter 2000). Although the existence of trait-environment relationships is not sufficient to prove that adaptive processes have driven radiation, the absence of such relationships suggests that adaptation in the traits measured did not play an important role. We focused our attention on two independent plant radiations within a biodiversity hot spot and asked whether we could detect associations between functional traits with presumed growth or fitness consequences and environments finely characterized by climatic variables.

We detected two or more environmental associations in both genera for nearly every trait, although we failed to detect any significant environmental associations for FWC in *Protea*. Because our environmental variables are characteristic of entire sites, the relationships we detect reflect associations between population means and the environments in which those populations are found. Since our model includes a phylogenetic random effect, the associations are also more likely to reflect similar environmental associations than common ancestry. We acknowledge that the mere existence of environmental associations provides only suggestive evidence for adaptation. The associations could reflect phenotypic plasticity in response to local conditions, or they could arise by chance. For example, trait differentiation associated with isolation by distance will lead to trait-environment associations along any environmental axis that covaries with distance (Reich et al. 2003). Nonetheless, our previous work in *Protea* (Carlson and Holsinger 2010; Prunier et al. 2012) provided evidence that several of the trait-environment relationships identified here represent adaptive responses to environmental gradients in a smaller subclade. Similarly, work by Martinez-Cabrera and Peres-Neto (2013) suggests that the rapid radiation within *Pelargonium* is associated with ecological differentiation along the summer precipitation gradient.

Together these results suggest a role for environmental adaptation in the radiations in *Protea* and *Pelargonium*, in keeping with theory and work in other rapidly diversifying clades. A number of studies have detected some manifestation of phenotype-habitat relationships in adaptive radiations, including (but not limited to) classic cases in *Anolis* lizards, Darwin's finches, the Hawaiian silversword alliance, and the Hawaiian lobeliads, each with consequences for function or fitness of the individual (Grant et al. 1985; Losos 1990; Robichaux et al. 1990; Givnish et al. 2004).

Do Parallel Radiations Have Parallel Responses?

The species of *Protea* and *Pelargonium* that we studied occupy broadly similar environments, and we might expect similar trait-environment relationships if environmental

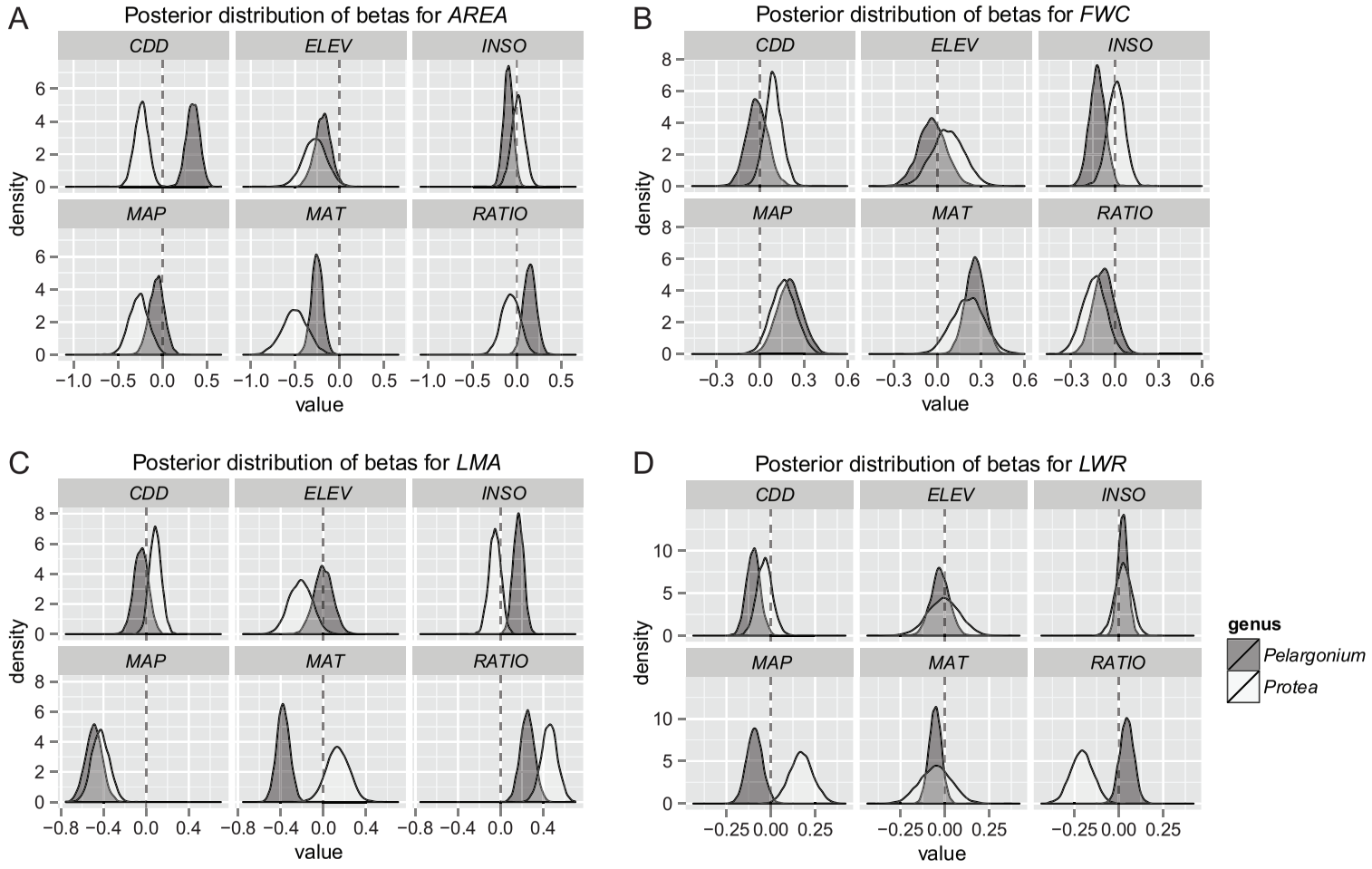

Figure 2: Posterior distributions for the regression coefficients (slopes) of our trait-environment associations for *Protea* (white) and *Pelargonium* (gray), from which we sampled to compare slopes between the two genera. Individual panels are for each plant trait against all six environmental covariates: *A*, canopy area; *B*, leaf fresh water content (FWC); *C*, leaf mass per area (LMA); and *D*, leaf length–to–leaf width ratio (LWR). See table 1 for additional abbreviations of elements and covariates.

adaptation played a role in shaping them. At the same time, the two genera also fill different niches in the communities in which they are found. Most *Protea* species are large, woody, evergreen shrubs and visually dominate the landscape, while *Pelargonium* are smaller shrubs or subshrubs, geophytes, either deciduous or evergreen, and often only seasonally apparent. Even if the macroclimates of the sites where they occur are similar, the microclimates, as well as other environmental factors such as soil fertility, that each genus experiences may be very different. In instances where *Protea* and *Pelargonium* show similar trait-environment relationships, those relationships may be generalizable to the entire fynbos biome (and possibly beyond). Where they show different relationships, however, those relationships may depend on details of growth form and life history within each group of plants (Adler et al. 2014) or on differences in the fine-scale environments each genus occupies.

In both genera we found that sclerophyllous leaves (high LMA) are associated with sites having lower rainfall and more seasonal rainfall (table 2). These associations are also consistent with worldwide analyses suggesting that sclerophylly is often an adaptation to areas of low precipitation (Wright et al. 2005; Poorter et al. 2009). Relationships with rainfall seasonality may be more specific to the CFR or areas dominated by Mediterranean climates in general, reflecting an association of high LMA with drought. The stronger relationship in *Protea*, for example, is consistent with the expectation that high LMA will be more strongly favored in plants with evergreen leaves persisting through a drought than in those that are largely drought-deciduous.

In contrast, we found that plant size (as indexed by canopy area) is associated with shorter periods of drought in *Protea* and longer periods of drought in *Pelargonium*. While the relationship in *Pelargonium* seems counterintuitive, it may reflect a drought-avoidance strategy in which many members of the genus grow quickly when resources are available and drop their leaves to avoid drought (Kumagai and Porporato 2012).

The differing associations between LWR and MAP are more difficult to understand. In *Protea* the positive association is consistent with expectations. Yates et al. (2010) postulated that narrow leaves (high LWR) allow plants to increase transpiration and nutrient accumulation when water is plentiful. In *Pelargonium*, however, interpretation of this relationship is confounded by leaf shape variation. Our measure of leaf width is the diameter of the largest circle that can be inscribed in the outline of a leaf such that low values can reflect either narrow leaves or broad leaves that are lobed or dissected. Although functional leaf width does not describe dissectedness, lobing, or venation, these traits are highly variable and important in *Pelargonium* leaf morphology and physiological function (Jones et al. 2009; Nicotra et al. 2011). If functionally narrow leaves are also highly dissected, the association of functionally narrow leaves in *Pelargonium* with low MAP may reflect an association between leaf dissection (which we did not measure in this study) and aridity.

Evolutionary Patterns in Trait Responses

It is widely accepted that selection favors individuals well suited to their environment; individuals with traits and trait combinations that increase fitness will be more likely to survive and produce offspring with like-adapted traits, if these are heritable (Marks and Lechowicz 2006; Poorter et al. 2008). We have demonstrated that parallel evolutionary radiations, with respect to biome and geological time, can exhibit both similar and disparate trait responses to environmental variables. Because *Protea* and *Pelargonium* fit into the fynbos community so differently and because their life histories and morphologies are so different, trait-environment relationships that are consistent across them may be generalizable across all broad-leaved plant groups in the fynbos of South Africa and perhaps across plant groups in Mediterranean-dominated climate regions.

Given the extreme differences in life form and position within the community in our two genera, we were surprised at the relative lack of evidence for discordant trait-environment associations, where only two had strong support for opposing signs. The lack of discordance might suggest that there are a few generalizable relationships across the fynbos and with other worldwide trends; for example, LMA is negatively associated with MAP in *Protea*, *Pelargonium*, and the worldwide LES (Wright et al. 2004, 2005). Nonetheless, the posterior probability of conflict was higher than that of agreement for half of the relationships, and these points of conflict also disagree with patterns at the global scale. We could not detect a relationship between LMA and MAT in *Protea*, and the relationship in *Pelargonium* is negative, while a recent meta-analysis on a global scale (Moles et al. 2014) found a positive relationship (a negative relationship with specific leaf area, the inverse of LMA). It is clear that although some trends may be consistent across groups within an individual biome (in our case, the fynbos), the relationship of within-biome patterns to those across biomes remains uncertain. In particular, it is difficult to anticipate when within-lineage and within-biome patterns of trait-environment associations will hold across lineages and biomes and when they will fail.

Conclusion

To the extent that evolutionary radiations are adaptive and adaptation involves responses to the physical environment, the history of adaptation may be reflected in contemporary associations between functionally significant traits and the environment. Here we identified a number

of such associations in two parallel plant radiations in the genera *Protea* and *Pelargonium*. We use an approach that allows us simultaneously to consider the association of multiple environmental factors with multiple traits. By including multiple environmental factors in the analysis, we can begin to distinguish direct associations from those that arise indirectly because of associations with other factors, as in selection gradient analysis (Lande and Arnold 1983). Similarly, by including multiple traits as a single response, we begin to account for the organismal context in which trait associations are expressed. It is, after all, whole organisms that respond to their environment as an integrated unit. The differing relationships in *Protea* and *Pelargonium* for canopy area with drought days and for LWR and precipitation, for example, illustrate that unmeasured aspects of plant life history may have a profound influence on individual trait associations.

Our analyses also showed that many trait-environment associations can be detected within evolutionary radiations of *Protea* and *Pelargonium*, consistent with a role for environmental adaptation. More importantly, our analyses suggested that while some of these associations may be repeatable within the fynbos, others depend on aspects of life history that differ markedly across plant groups or are not generalizable to global-scale patterns. In short, patterns that emerge in studies spanning many biomes and including many distantly related species may not reflect those detected within smaller clades, suggesting that the processes responsible for global-scale associations may be different from those that produce such associations within more closely related groups of species.

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Appendix from N. Mitchell et al., "Functional Traits in Parallel Evolutionary Radiations and Trait-Environment Associations in the Cape Floristic Region of South Africa" (Am. Nat., vol. 185, no. 4, p. 525)

Additional Variable Correlations

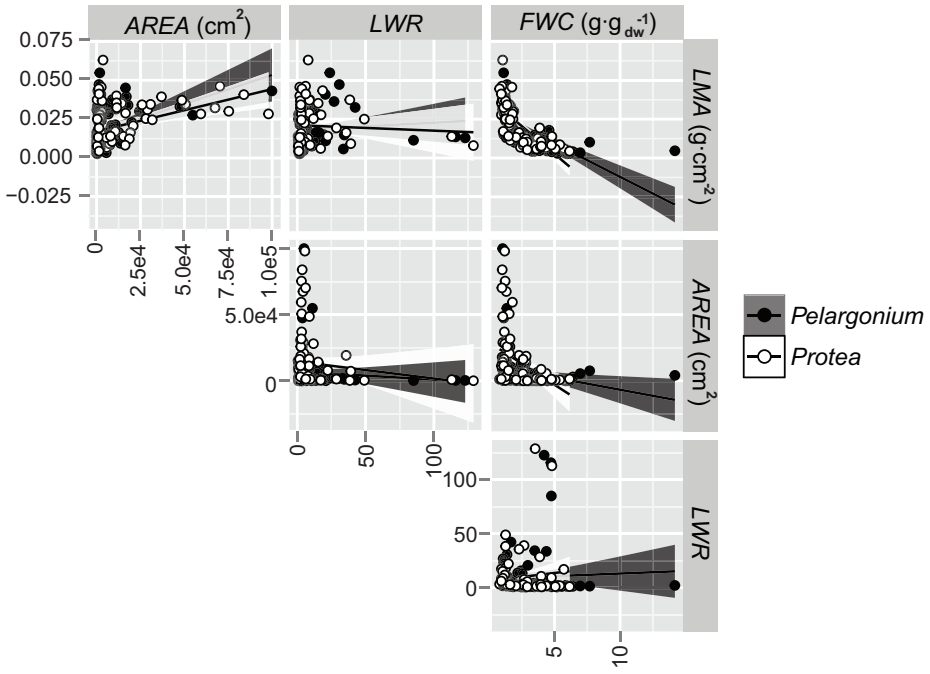

Figure A1: Trait-trait correlations in *Protea* and *Pelargonium*. Values are population means (not standardized) with units and abbreviations as described in table 1. Plots were created using the package ggplot2 in R, version 2.15.2. Trend lines shown are simple regressions (method = lm), where the shaded region indicates the 95% confidence intervals. Plots are to be used for comparing general trends in trait values across genera.

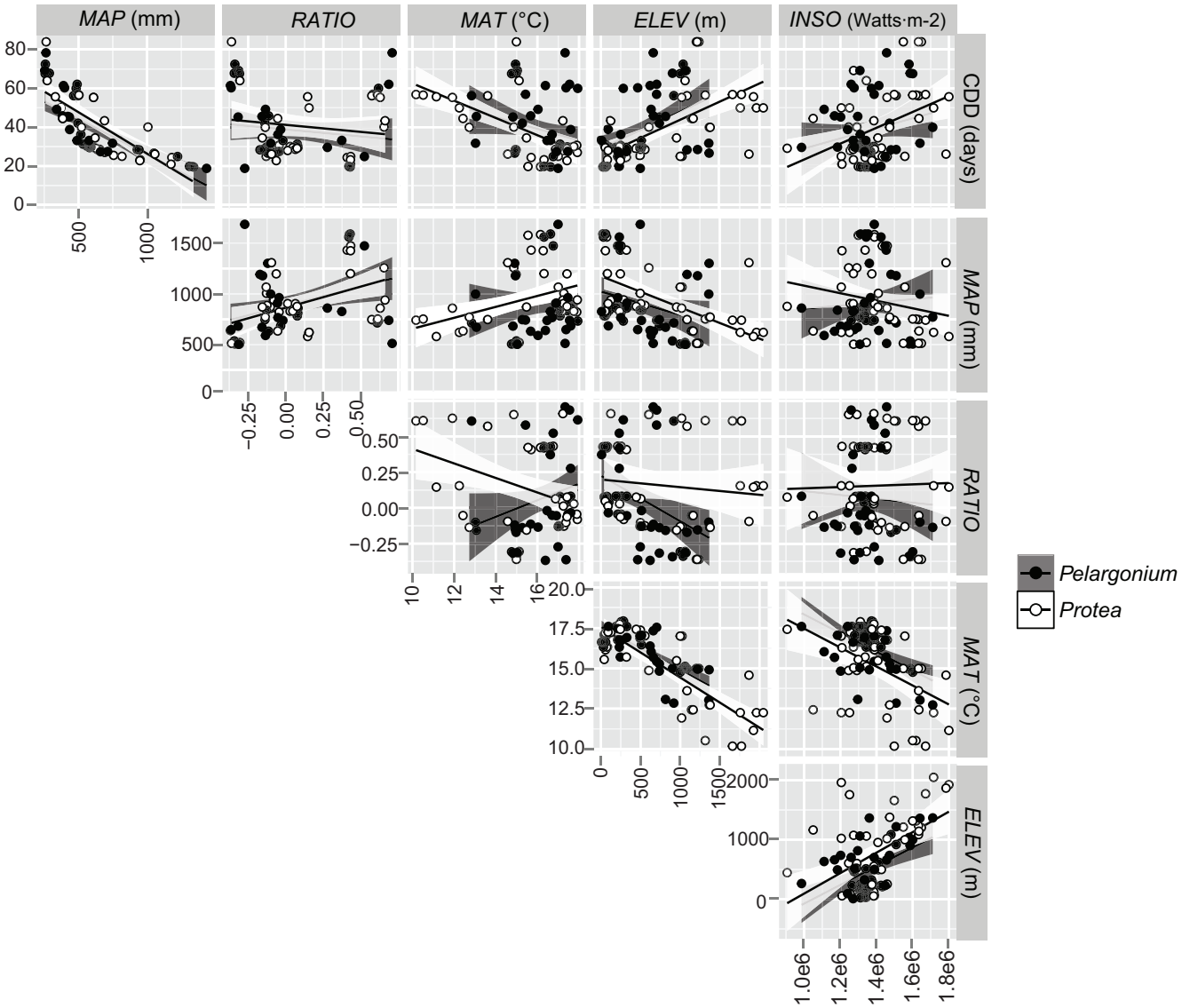

Figure A2: Environment-environment correlations in *Protea* and *Pelargonium*. Values are population means (not standardized) with units and abbreviations as described in table 1. Plots were created using the package ggplot2 in R, version 2.15.2. Trend lines shown are simple regressions (method = lm), where the shaded region indicates the 95% confidence intervals. Plots are to be used for comparing general trends in environmental values across genera.

Author Contributions

N. Mitchell assisted in data collection and cleanup, ran the analyses, and wrote the majority of this manuscript. T.E. Moore assisted in data collection and cleanup and writing. H.K. Mollmann extracted environmental data. J.E. Carlson was crucial in data collection, K. Mocko, H. Martinez-Cabrera, and C. Adams all assisted in data collection. J.A. Silander, C.S. Jones, and C.D. Schlichting provided substantial feedback, discussion, and revision. K.E. Holsinger wrote the initial modeling code and wrote portions of the methods.

Additional online supplementary material for Chapter 1 may be found at:

Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.sc286

Chapter 2:

Anchored phylogenomics improves the resolution of evolutionary relationships in the rapid radiation of *Protea* L.

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RESEARCH ARTICLE

AMERICAN JOURNAL OF BOTANY

Anchored phylogenomics improves the resolution of evolutionary relationships in the rapid radiation of *Protea* L.[1]

Nora Mitchell[2,5], Paul O. Lewis[2], Emily Moriarty Lemmon[3], Alan R. Lemmon[4], and Kent E. Holsinger[2]

PREMISE OF THE STUDY: Estimating phylogenetic relationships in relatively recent evolutionary radiations is challenging, especially if short branches associated with recent divergence result in multiple gene tree histories. We combine anchored enrichment next-generation sequencing with species tree analyses to produce a robust estimate of phylogenetic relationships in the genus *Protea* (Proteaceae), an iconic radiation in South Africa.

METHODS: We sampled multiple individuals within 59 out of 112 species of *Protea* and 6 outgroup species for a total of 163 individuals, and obtained sequences for 498 low-copy, orthologous nuclear loci using anchored phylogenomics. We compare several approaches for building species trees, and explore gene tree–species tree discrepancies to determine whether poor phylogenetic resolution reflects a lack of informative sites, incomplete lineage sorting, or hybridization.

KEY RESULTS: Phylogenetic estimates from species tree approaches are similar to one another and recover previously well-supported clades within *Protea*, in addition to providing well-supported phylogenetic hypotheses for previously poorly resolved intrageneric relationships. Individual gene trees are markedly different from one another and from species trees. Nonetheless, analyses indicate that differences among gene trees occur primarily concerning clades supported by short branches.

CONCLUSIONS: Species tree methods using hundreds of nuclear loci provided strong support for many previously unresolved relationships in the radiation of the genus *Protea*. In cases where support for particular relationships remains low, these appear to arise from few informative sites and lack of information rather than strongly supported disagreement among gene trees.

KEY WORDS anchored phylogenomics; coalescence; phylogenetics; Proteaceae; radiation

Evolutionary radiations, which are typically associated with rapid bursts of diversification into many species and morphological forms, provide ideal systems for studying evolution. They can be found at both deep and shallow taxonomic levels, from the origin and explosion in diversity of all flowering plants (Crepet, 2000) to individual families (e.g., Restionaceae; Linder, Eldenas, and Briggs, 2003), groups within families (e.g., Hawaiian silversword alliance; Baldwin and Sanderson, 1998), genera (e.g., *Pelargonium;* Bakker et al., 2004), and even subclades (e.g., the white proteas; Prunier and Holsinger, 2010). Robust estimates of relationships among taxa are a prerequisite for studying the morphological, ecological, and often cytological diversity in radiating groups and understanding trait evolution. However, the rapid evolution that makes these systems so interesting also makes it difficult to build well-resolved phylogenies (Knowles and Chan, 2008). Rapid radiation leads to many short branches with few nucleotide differences reflecting shared ancestry. Gene trees may not reflect the same history as the species tree because of incomplete lineage sorting (ILS), where alleles coalesce prior to the splitting of species, gene duplication, or loss, or because of hybridization (Maddison, 1997). Empirical data sets in many systems have found evidence for discordance among nuclear loci (reviewed in Degnan and Rosenberg, 2009), and in particular, several studies have highlighted the high frequency of gene tree discordance in more recent radiations (Knowles, 2009;

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Stephens et al., 2015a, 2015b). Here, we use a rapid plant radiation and several methods to generate species-level phylogenies and assess the influence of possible causes of gene discordance.

Novel methods for acquiring massive amounts of DNA sequence data for building nuclear species trees have resulted in more strongly supported trees at both deep (Lemmon et al., 2012; Prum et al., 2015; Xi et al., 2014) and relatively shallow time scales (Pyron et al., 2014; Brandley et al., 2015; Nicholls et al., 2015; Ruane et al., 2015; Shen et al., 2015; Stephens et al., 2015a, 2015b). Using many presumably unlinked genetic loci not only has the potential to vastly increase the number of phylogenetically informative characters, it also lessens the risk of being misled by a small set of gene tree histories that fail to reflect the species tree (Leaché and Rannala, 2010). There are many ways to capture huge numbers of loci from genomic DNA, including targeting loci designed across taxa (Faircloth et al., 2012; Lemmon et al., 2012; see Lemmon and Lemmon, 2013 for a review). In anchored phylogenomics (Lemmon et al., 2012), probes are designed across the taxonomic group of interest (in this case, angiosperms) from known sequence data and used as baits to capture the target and flanking DNA regions in nonmodel organisms (Buddenhagen et al., 2016). This allows DNA sequences to be collected from hundreds of genes without requiring expensive and time-consuming preliminary experiments to identify target DNA sequences.

The huge influx of data associated with phylogenomics has, unsurprisingly, highlighted issues concerning the best approach for phylogenetic inference. These issues mainly concern computational feasibility (how to handle huge data sets) and the underlying assumptions and statistical efficiency of alternative approaches (concatenation vs. species tree approaches). Concatenation methods treat the sequence data as a single set of characters, but implicitly assume that all genes share a common history. If incomplete lineage sorting is common, as is expected for radiations, concatenation methods may be statistically inconsistent (Kubatko and Degnan, 2007; Roch and Warnow, 2015). In contrast, species tree approaches often take individual gene tree information into account (Bryant, 2003), using inferential methods that attempt to address discordance introduced by ILS, as well as discordance arising from lack of resolution. These include "shortcut" methods, such as MP-EST and NJst (Liu et al., 2010; Liu and Yu, 2011) and a new generation of species tree inference methods, such as ASTRAL-II (Mirarab and Warnow, 2015) and SVDquartets (Chifman and Kubatko, 2014), which each implement different, simplified models accounting for discordance due to ILS. Empirical and simulation studies have shown that the choice of methods (concatenation or species tree) may have a substantial (Xi et al., 2014) or little effect (Chou et al., 2015; Tonini et al., 2015) on the estimation of phylogeny, often depending on the amount of ILS. In empirical data sets, it is therefore necessary to employ both kinds of methods to either verify congruence or explore reasons for topological differences.

The genus *Protea* L. (Proteaceae) is a well-studied plant radiation, consisting of approximately 112 species, with a fairly recent crown age of 5–18 mya (Sauquet et al., 2009). The genus has its center of diversity and origin in the Cape Floristic Region (CFR) of South Africa, a biodiversity hotspot (Myers et al., 2000; Valente et al., 2010). It is an iconic lineage in the CFR, and is 1 of 30 groups contributing to the bulk of the extraordinary plant diversity in this region (>9000 species) (Goldblatt and Manning, 2000; Linder, 2005). *Protea* are all evergreen, sclerophyllous shrubs, but their growth forms range from low-growing individuals to small trees, and they show substantial diversity in leaf shape and size. Species differ in many functional traits, and several are correlated with important environmental variables such as seasonality and mean annual precipitation and temperature (Mitchell et al., 2015). Common-garden experiments have demonstrated both inter- and intraspecific adaptive differences in physiological and functional traits (Carlson et al., 2011; Prunier et al., 2012; Carlson et al., 2015).

Previous phylogenetic analyses of this genus used only a few molecular markers: the nuclear ribosomal DNA region ITS, a set of plastid noncoding regions, the nuclear gene ncpGS, and 138 AFLP loci (Valente et al., 2010; Schnitzler et al., 2011; the latter species tree approach used the same data without AFLPs and included additional taxa). Although these analyses identified a few well-supported clades, many relationships were poorly resolved. In addition, some groups that have long been recognized based on morphological characters (Rourke, 1982; Rebelo, 2001) are not supported in the published phylogenies. For example, *P. laurifolia* and *P. neriifolia* are morphologically very similar and replace one another geographically, yet molecular phylogenies suggest they are distant relatives (Valente et al., 2010; Schnitzler et al., 2011). Hybridization is also known to occur in *Protea* (Prunier and Holsinger, 2010), possibly contributing to regions of the tree with low support (Valente et al., 2010). To build a more-strongly supported phylogeny for *Protea* as a basis for future analyses of trait evolution, we collected samples from multiple populations throughout South Africa and used targeted sequencing techniques to greatly increase the number of DNA sequence markers available for phylogenetic inference.

We aim to (1) resolve relationships within the rapid, recent radiation of *Protea*; (2) compare widely used concatenation and species-tree approaches; and (3) explore the causes of differences in gene tree and species tree topologies.

MATERIALS AND METHODS

Taxon sampling—We collected DNA samples from fresh leaf tissue in the field from 2011–2014 using locations from the Protea Atlas database (http://www.proteaatlas.org.za/), live accessions at Kirstenbosch Botanical Gardens, and greenhouse-grown individuals derived from wild-collected seed (Prunier et al., 2012 Fig. 1; see Appendix S1 for a full list of species and voucher information in the Supplemental Data with this article). In all, our initial data set includes samples from 163 individuals collected from 65 species, including 6 outgroup species (*Serruria* and *Faurea*) and 59 *Protea* species (Appendix 1); DNA was collected into a concentrated CTAB solution (Doyle and Doyle, 1987).

These samples represent a substantial fraction of species in *Protea*: over half of the total species, and approximately 70% of the species found in South Africa (Rourke, 1982), but our own sampling is insufficient to build the most complete phylogeny possible with available DNA data. To supplement our samples, we used sequence data from Valente et al. (2010) and Schnitzler et al. (2011) as downloaded from TreeBase.org (S11132). The Schnitzler data set includes 32 additional species of *Protea* and 7 outgroup taxa with sequence information from 4199 additional bases in plastid noncoding regions, as well as the nuclear genes ITS and ncpGS. This AUGMENTED data set thus uses the sequence data from Schnitzler et al. (2011), but builds on trees constructed using our samples. We used this data set to construct a phylogenetic estimate for *Protea* that includes 91 of the 112 extant taxa plus 13 outgroup taxa (see Results section).

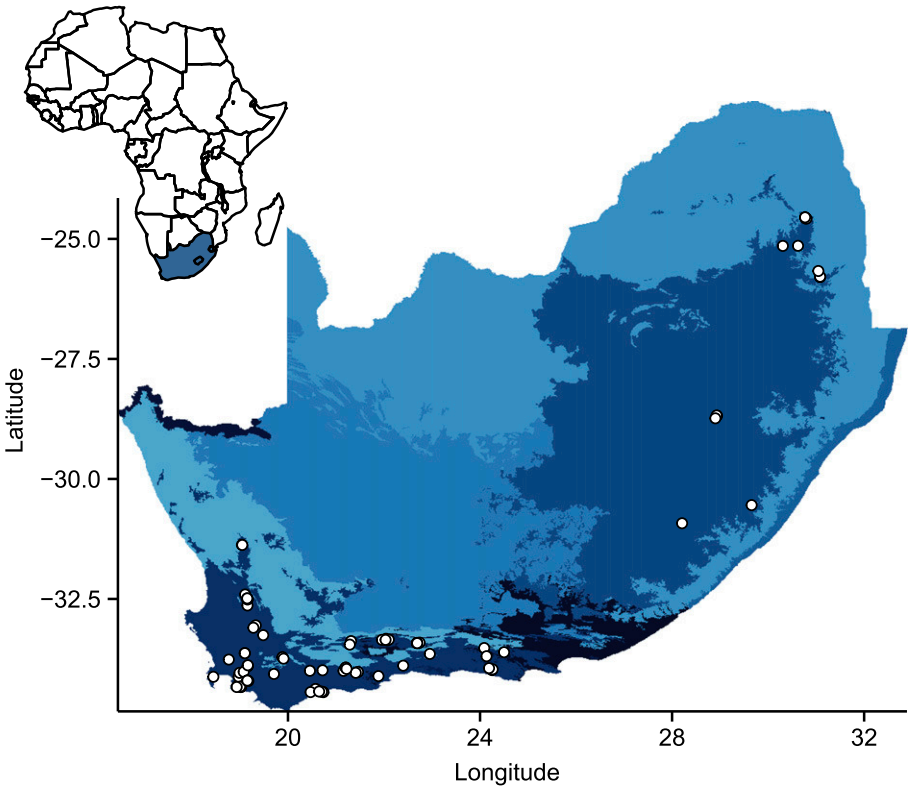

FIGURE 1 Sampling map for wild-collected *Protea* species across South Africa. Shades indicate biomes as defined by Mucina and Rutherford (2006). Most samples were collected in the fynbos biome, with some in the northeastern part of the country in grassland biomes. See Appendix S1 for voucher information and latitude and longitude data.

DNA extraction, target enrichment, and sequencing—We extracted DNA from the CTAB-preserved leaf tissue using a modified CTAB approach (Doyle and Doyle, 1987). Further molecular work was conducted at the Center for Anchored Phylogenomics (www.anchoredphylogeny.com) at Florida State University. The lack of polyploidy in *Protea* (Oberlander et al., 2016) facilitates the assembly of anchored phylogenomics data and avoids the possibility of complications due to whole-genome duplication. Sequences for anchored nuclear loci were obtained from 498 low-copy orthologous regions identified and designed across angiosperms, 482 based on the orthologs of Duarte et al. (2010), and an additional 16 genes identified as important in selenium tolerance as in Buddenhagen et al. (2016) using the general methods of Lemmon et al. (2012). Loci numbers are not identical to Buddenhagen et al. (2016), but *Arabidopsis* gene identifiers match (Appendix S2). In short, extracted DNA was sonicated via a Covaris E220 Focused-ultrasonicator (Woburn, MA) to obtain 300–800 bp fragments. Libraries were prepared and indexed on a liquid-handling robot (Beckman-Coulter Biomek FXp, Brea, California) using the protocol of Meyer and Kircher (2010). One modification of the protocol included a size-selection step, removing fragments <200 bp in length, after blunt-end repair using Solid Phase Reversible Immobilization (SPRI) select beads (Beckman-Coulter). After indexing, samples were pooled in equal quantities (16–18 samples per pool), and each pool was enriched using an Agilent Custom SureSelect kit (Agilent Technologies, Lexington, Massachusetts). Enrichment pools were run in equal quantities for sequencing on replicate PE150 Illumina HiSeq2500 lanes (typically three pools, which included ~48 samples per lane). Sequencing was performed in the Translational Science Laboratory in the College of Medicine at Florida State University.

Bioinformatics processing—Reads were processed following Prum et al. (2015), Ruane et al. (2015), and Pyron et al. (2016). For paired-read merging, the probability of overlapping to a given degree by chance was calculated, and read pairs with significant matches were merged (see Rokyta et al., 2012). Base-specific quality scores were used to reconcile differences and were combined to produce quality scores for the merged reads. For the target regions, divergent reference assembly was used to map reads to the probe region sequences for *Arabidopsis thaliana, Aquilegia coerulea*, and *Nelumbo nucifera*, and de-novo assembly was then used to extend these to flanking regions (see Prum et al., 2015 for details). A coverage filter removed low-coverage contigs (<20 reads) to remove reads from potential cross-contamination. To assess putative orthology among consensus sequences at each locus, pairwise distances between two sequences were computed using the percent of 20-mers found in common between each pair of sequences. A Neighbor-Joining algorithm was then used to cluster sequences based on these pairwise distance measures (see Prum et al., 2015 for details). Two alleles were phased per consensus sequence following Pyron et al. (2016), using a Bayesian approach that estimates the posterior distribution of phasing solutions from assembled reads.

Sequences in each orthologous set were aligned using MAFFT v7.023b (Katoh and Standley, 2013). Alignments were trimmed by identifying "good sites" (sites where the most common state was present in >50% of the sequences), masking 20 bp regions that contained <14 good sites, and removing sites with <240 unmasked bases. After the pipeline of filtering, orthology, trimming, and masking, the sequence data consisted of both target regions and variable flanks for 498 target loci.

Phylogenetic analysis—Our first goal was to build species-level phylogenies for *Protea* using two concatenation and two species tree methods, as well as gene trees for individual loci (Table 1). Figures for trees were created using TreeGraph2 (Stöver and Müller, 2010).

We used four different sets of data derived from the raw sequence data in our analyses (Table 2). The complete set (hereafter referred to as the COMPLETE data set) includes 163 individuals (from 59 species of *Protea* and 6 outgroup species) and sequences from both alleles for up to 498 loci. Not all loci were captured for all taxa; in this data set, nucleotides at these loci were coded as missing values (Appendix S1). Analysis of this data set allows us to assess monophyly of most species-level taxa in our data set, and can also be used in multi-individual modes in ASTRAL-II and SVDquartets. To reduce the computational burden for other analyses and to build species-level

TABLE 1. Summary of tree-building methods. "Gene Trees" indicates that all samples were used as terminals, "Full Trees" means that species were used as terminals.

Method	Input	Output	Methodology
Gene tree building			
RAxML	Sequence	Gene Trees	Concatenated
Species tree building			
RAxML	Sequence	Full Tree	Concatenated
MrBayes	Sequence	Full Tree	Concatenated
SVDquartets	Sequence	Full Tree	Species Tree
ASTRAL-II	Gene Trees	Full Tree	Species Tree

phylogenies, we sampled species rather than individuals by creating consensus sequences coded with ambiguities for each taxon using BioEdit v7.2.5 (Hall, 2013), reducing the number of taxa to the 65 identified species (hereafter referred to as the CONSENSUS data set). We refer to the COMPLETE data set when possible, but use CONSENSUS for making tree comparisons to be consistent with the sequence data used. Taking species-level consensus sequences may mask information, especially if species are not monophyletic. To check this, we also created a ONEPER data set that contains one arbitrarily selected sequence per species, rather than a consensus across all sequences for each species (see Appendix S3 for details on the ONEPER data set and analyses).

Six individuals (one *P. grandiceps*, one *P. nubigena*, one *P. recondita*, and three *Serruria* samples) failed to recover many loci, and likewise, over a quarter of the loci were not recovered for many individuals (Appendix S1, Appendix S2). We trimmed the CONSENSUS data set to obtain a REDUCED data set that includes 60 taxa (57 *Protea*, three outgroup) and 354 loci with complete data. This was necessary for comparing gene tree topologies, because all of the tips in the trees must be the same with no missing taxa. Finally, the aforementioned AUGMENTED data set includes taxa and sequence data from Schnitzler et al. (2011). To assess the information found in each site, we computed the number of parsimony informative sites at each locus for the COMPLETE, CONSENSUS, and ONEPER sequences using the pis() function in the R package 'phyloch' (Heibl, 2013).

Individual gene trees for all 498 loci in the COMPLETE and ONEPER data sets were obtained in RAxML v8.3.17 (Stamatakis, 2014) using a GTRGAMMA model and 100 bootstrap replicates. For each locus, we saved 100 bootstrap replicates for each of the gene trees and used them in the subsequent ASTRAL-II analysis. We also saved the best maximum likelihood gene trees from the COMPLETE analysis and used these to compute distances and internode certainty (IC) values on our species trees in RAxML (Salichos et al., 2014). Internode certainty takes into account the frequency of the most common taxon bipartition in comparison to the most observed conflicting bipartition.

TABLE 2. Summary of data sets used. Note that not all loci are represented by all species in the data set except in the case of the REDUCED set. Tips indicate the terminals in the tree: sequences used were either associated with alleles, individuals, or species (see brief description).

Data set	# Loci	# Tips	Description
COMPLETE	498	163	All alleles and individuals
CONSENSUS	498	65	Species-level consensus
ONEPER	498	65	One individual selected per species
REDUCED	354	60	All loci found in all CONSENSUS species
AUGMENTED	3	99	Backbone from this study, sequences from Schnitzler et al. (2011)

Analyses of concatenated data were conducted in RAxML v8.3.17 (Stamatakis, 2014), also using a GTRGAMMA model and 100 bootstrap replicates. Analyses were conducted on the CONSENSUS and ONEPER data sets (all 274,405 bp from the 498 loci as one sequence per sample, 65 species total, with separate partitions for each locus) to obtain a species tree estimated using concatenation. We did not partition by codon position because identifying coding regions and codon position is difficult for this type of data set. To check for species monophyly, we ran RAxML over the COMPLETE data set with the same settings. We also conducted a concatenated, unpartitioned Bayesian analysis using the CONSENSUS and ONEPER sequences in MrBayes v3.2.1 (Ronquist and Huelsenbeck, 2003) MPI version, under a GTR +I +G model with four chains for 5 million generations, thinned to save one sample every 1000 generations. Parameters were visually checked in Tracer to confirm convergence, and a consensus tree (plus other compatible groupings) was computed in PAUP* after a 500 tree burn-in and used as a species tree for further analyses. We did not use MrBayes to check for species monophyly because of the size of the data set.

ASTRAL-II (Mirarab et al., 2014, Mirarab and Warnow, 2015) estimates a species tree from input gene trees, and has been shown to be statistically consistent under the multispecies coalescent model. ASTRAL-II finds the species tree that maximizes the number of embedded quartet trees in the given gene trees; it works efficiently by limiting the number of bipartitions explored to those included in the supplied gene trees. Additionally, ASTRAL-II is capable of taking information from bootstrap replicates of these gene trees, as well as including multiple individuals per species. We employed the bootstrapping method in ASTRAL-II v4.7.9 to estimate a species tree using this coalescent-based approach, as well as the multi-individual feature using the /multiind branch of the ASTRAL-II GitHub repository (https://github.com/smirarab/ASTRAL/tree/multiind). This option allows for a species-level estimation rather than building a tree with multiple accessions per species. Best trees and bootstrap replicates were estimated in RAxML separately for each locus in the COMPLETE data set. These best trees, bootstrap files, and a species-to-allele file were provided for each locus and run for 100 bootstrap replicates. We also ran ASTRAL-II using the best trees and bootstrap files from the ONEPER and CONSENSUS data sets to obtain species trees and the COMPLETE best trees and bootstrap files to check for species monophyly.

SVDquartets (Chifman and Kubatko, 2014) is a recent quartet-based species tree method that is robust to ILS given data that is reasonably clock-like. This method treats each single nucleotide polymorphism (SNP) as an independent sample from a species tree with a coalescent history within species. It produces a species tree estimate, rather than estimates of individual gene trees. We performed the SVDquartets analysis on the COMPLETE data set in the test version of PAUP* 4.0a146 (Swofford, 2003) using the QFM matrix agglomeration method (Reaz et al., 2014). We used the multispecies coalescent approach with the species-membership partition, searching 1 million quartets, and did a bootstrap analysis of 100 replicates. For the CONSENSUS and ONEPER data sets we used the same settings, but did not include the species-membership partition scheme. To check for species monophyly, we searched a reduced set of 10,000 quartets because of the increased number of tips in the tree. Bootstrap trees for each data set were saved and an SVDquartets consensus tree was computed in PAUP*.

Species topologies as constraint trees—SVDquartets and ASTRAL-II produce species-level phylogenies that take into account multiple individuals per species. To estimate branch lengths, the 80% majority rule topologies from each of the ASTRAL-II and SVDquartets trees were input as constraint trees and run in RAxML with the CONSENSUS sequence data. The topology from the best RAxML concatenated tree has maximum-likelihood lengths associated with each branch.

We used the species trees as backbones on which to place the 39 additional species included in Schnitzler et al. (2011), the AUGMENTED data set. We did this by removing species for which Schnitzler et al. (2011) had no data and using the remainder to construct 80% majority-rule consensus trees from the bootstrap replicates for the SVDquartets, RAxML, and ASTRAL-II trees. For the MrBayes species tree, we calculated the 80% SVDquartets majority-rule tree after a 500-tree burn-in in the second run of our analysis, which converged more quickly. We then used these backbones as constraint trees in RAxML for analyses of the concatenated sequence data (4199 bp) from Schnitzler et al. (2011) under the GTRGAMMA model. This method may be problematic where our data and the sequences from Schnitzler et al. (2011) suggest different topologies, but it can provide a rough estimate of placement of additional species for which anchored phylogenomics data are not available.

Relevant alignment and tree files are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.vj32s. Raw sequence reads are deposited in the NCBI SRA BioProject ID PRJNA354967, SRA study SRP093931.

Hypothesis testing: poor support due to few changes or ILS—We used the REDUCED data set to estimate distances between gene and species trees. Each of the 354 individual gene alignments was run in RAxML (using the above settings) to obtain the best and bootstrapped gene trees. We also generated 354 random topologies in PAUP*, using the proportional-to-distinguishable model, to compare the distribution of gene tree distances to those of randomly generated trees, and we simulated 354 gene trees from the ASTRAL-II species tree in the program COAL, which is used for computing gene tree distributions (Degnan and Salter, 2005), using branch lengths of one (branch lengths are equal to the number of generations / (2 * the effective population size)). Branch lengths of 0.5 and 0.2 did not significantly affect simulated tree topologies.

To determine which trees were most similar and which may be most reliable, we compared all tree topologies using an adjusted Robinson-Foulds (RF) distance. We calculated raw distances (RF) using RF.dist() from *phangorn* (Schliep, 2011) in R v3.1.3 (R Core Team, 2015) and adjusted RF distances as RFadj = RF/(2n−6) where n is the number of nodes on the tree (Steel and Penny, 1993). The RFadj values can range from zero (topologically identical) to one (completely dissimilar). We calculated several sets of distances: (1) among individual RAxML gene trees, (2) among random trees, (3) among trees simulated from the coalescent, (4) between individual gene trees and species trees produced via concatenation or species tree building methods, and (5) among the species trees. We compared the distributions of RFadj between different sets of trees by performing two-tailed T-tests in R. We adjusted the sample size in our T-tests to be more conservative, using only the number of trees compared, not the number of pairwise comparisons, which are nonindependent.

Low levels of support at any particular branch of a species tree could reflect either short branches or a balance between strongly supported but conflicting gene histories (i.e., ILS). To distinguish between these possibilities in clades of particular interest, we took the existing, fully bifurcating ASTRAL-II tree topology and constructed trees with alternative resolutions to match the different species trees from the COMPLETE analysis ("A = ASTRAL-II", "B = SVDquartets", "C = RAxML/MrBayes") at only branches of interest. We then measured distances from both the RAxML "best" gene trees and the bootstrap replicates for each of those gene trees to the "A", "B", or "C" species tree topologies. We call these ad hoc tests "alternative placement tests". If poor support is the result of a small number of changes, we expect that the distances from gene trees to each species tree will be sampled from a single underlying distribution. If poor support is the result of balance between strongly supported conflicting topologies, one set of genes will have a shorter distance to one topology, and another set will have a shorter distance to an alternate topology. We are particularly interested in the relative placement of *P. repens*, because it is the most widespread South African endemic in *Protea*, and much recent work has focused on intraspecific variation and local adaptation in this species at the morphological, physiologically, genomic, and transcriptomic levels (Akman et al., 2015; Carlson et al., 2015; Prunier et al., personal communication). In our species trees, *P. repens* is sometimes sister to *P. rupicola*, and other times has a more complicated relationship, leading us to focus on the placement of these two species as a case study.

Hypothesis testing: poor support due to hybridization—If hybridization has caused a lack of confidence in relationships in the phylogeny of *Protea*, we would expect to see evidence of reticulation in areas of the tree associated with low bootstrap support values. We built a phylogenetic network to visually identify regions of the *Protea* phylogeny possibly associated with hybridization, which could potentially generate observed conflict among gene trees, in SplitsTree4 (v 4.13.1) (Huson and Bryant, 2006). We used the COMPLETE sequences for this analysis, and we excluded outgroup species to emphasize ingroup relationships. We used the JC69 model to estimate species distance with the "NeighborNet" distance transformation. We ran SplitsTree4 on the ONEPER and CONSENSUS data sets using the same settings.

RESULTS

Target enrichment—We captured up to 498 loci across the 163 specimens, with both alleles assessed for each of our samples in the COMPLETE data set. The concatenated sequence contains 274,405 bp with an average locus length of 551 bp. The COMPLETE data set contained 67,677 parsimony-informative (PI) sites and an average of 139 PI sites per locus with 7.5% of characters coded as gaps/missing. When we create species-level CONSENSUS sequences, these numbers drop to 31,422 PI sites total and 66 PI sites per locus with 4.85% of data missing or coded as gaps. The ONEPER analysis had a total of 35,712 PI sites, with an average of 72 per locus and 7.5% of data coded as missing/gaps. The REDUCED data set had only 14,612 PI sites with an average of 41 per locus and only 1.3% missing data. See Appendix S2 for additional locus information.

Species monophyly—Phylogenetic tree topologies for the COMPLETE data set revealed monophyly for most species sampled, with a few exceptions (Appendix S4). The trees generated using the three

methods were fairly dissimilar as measured by the adjusted Robinson-Foulds distance: (RAxML-SVDquartets = 0.484, RAxML-ASTRAL-II = 0.413, SVDquartets-ASTRAL-II = 0.587). Notably, the two methods that incorporate the multispecies coalescent (SVDquartets and ASTRAL-II) were the most dissimilar when examining all individuals and all alleles per species. The main instances of consistent nonmonophyletic species were within the white protea clade, for which species and subspecies were highly mixed. Other regions included grades or very close placement rather than true monophyly (e.g., *P. piscina*), or divergent individuals with anomalous placement (e.g., *P. scolopendriifolia* 246, *P. cordata* 42B, *P. recondita* 58A, P. *burchellii* 1476).

Species-level phylogeny estimation—Tree topologies derived from concatenated vs. species tree strategies were fairly similar for the CONSENSUS data set, in terms of adjusted Robinson-Foulds distance with an average of 0.276 across the six pairwise comparisons. The two concatenated trees were topologically identical (RAxML-MrBayes = 0), while differences between the two species tree-build trees were greater (SVDquartets-ASTRAL-II = 0.290) (Fig. 2, Appendix S5). Comparisons across methodologies were more dissimilar (RAxML-SVDquartets = 0.381, RAxML-ASTRAL-II = 0.302). Results were similar if we used the COMPLETE data set (for SVDquartets-ASTRAL-II = 0.129) instead of CONSENSUS. Results for the ONEPER data set were qualitatively similar; see Appendix S3 for trees built using the ONEPER data set and comparisons across data sets. In spite of these differences, all four approaches produced trees that were much more similar to one another than any of the gene trees were to each other. They were also more similar to one another than any of the gene trees were to any of the species trees. We examined relationships further in all four species trees, but displayed the ASTRAL-II tree as a representative (Fig. 3). Although we cannot say that one tree is more accurate than another, ASTRAL-II uses bootstrapped gene trees, and topological differences among the species trees are relatively minor. The remaining trees are found in Appendix S5.

The species tree topologies in the COMPLETE data set all had three strongly supported clades, which are largely consistent with the previously published trees of Valente et al. (2010) and Schnitzler et al. (2011). These include the snow proteas plus *P. cynaroides*, which are sister to all remaining species within *Protea*, a large clade containing the non-Cape clade, rose, shale, penduline, and western ground proteas, and another clade largely containing the white, rodent, spoon-bract, bearded, dwarf-tufted, and eastern ground proteas. Morphologically defined clades within these large groupings in *Protea* do not consistently reflect evolutionary relationships among species either in previous studies or in our species trees (see Appendix S1 for classifications from Rebelo, 2001). There is also a lack of consistency and confidence in the placement of two species in particular: *P. repens* and *P. rupicola*, which we investigate further.

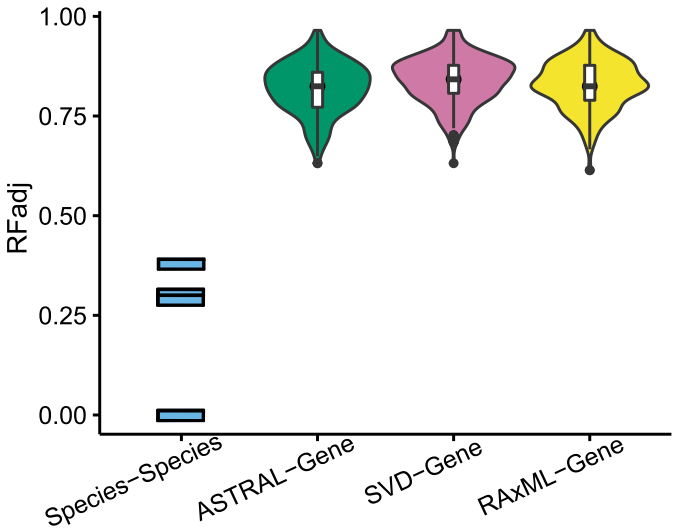

FIGURE 2 Adjusted Robinson-Foulds distances among the three CONSENSUS species trees (first column, shown as horizontal bars) and between each species tree and each gene tree.

The average bootstrap value across branches was 93% in the ASTRAL-II analysis and 90% for the SVDquartets analysis using the COMPLETE data sets. Values were lower using the CONSENSUS data sets (92% for ASTRAL-II and 86% for SVDquartets, 87% for RAxML). As is commonly seen, posterior support for branches in the CONSENSUS Bayesian analysis was higher than bootstrap support (an average posterior probability of 0.98 in the MrBayes analysis; Appendix S5). The COMPLETE ASTRAL-II tree had only 7 branches with less than 80% bootstrap support and a total of 12 with less than 95%; SVDquartets had 12 branches with less than 80% and 18 with less than 95%; CONSENSUS RAxML had 14 branches with less than 80% and 21 with less than 95%. MrBayes had 3 branches with less than 0.95 posterior probability, and 10 that were under 1.00.

We incorporated sequence data from Schnitzler et al. (2011) with our anchored phylogenomics set (AUGMENTED data set) using 80% majority rule consensus species trees as constraints. Our resulting species trees were quite different from the maximum clade credibility tree in Schnitzler et al. (2011; see their Figure S3), with an average RFadj value of 0.587 between it and the four constraint species trees (Appendix S6). The average RFadj value among our four species trees was 0.365, although trees built using the same method were not more similar as they were in analyses of the COMPLETE or CONSENSUS data sets.

Branch lengths across the phylogeny tended to be very short. Omitting branches associated with outgroup taxa, the average branch lengths for all four trees were consistent at 2.84×10^{-3} substitutions per site for internal branches. The minimum values ranged from 2.23×10^{-6} for the SVDquartets tree to 1.04×10^{-5} for the RAxML/MrBayes tree. The maximum branch lengths ranged from 0.0941 for the RAxML/MrBayes tree to 0.0947 in the SVDquartets tree (Appendix S7).

Hypothesis testing: poor support due to few changes or ILS?—Branches may have low support either because there is little information across all loci to resolve relationships (due to sites evolving too slowly or too rapidly, resulting in saturation) or because the different sets of genes have histories that are incompatible with species trees that either ignore gene tree topological variation, or account for it with ILS alone. The 50% SVDquartets consensus tree from the 354 REDUCED set best gene trees resulted in a topology with only two ingroup branches resolved, indicating either that individual gene trees lack sufficient information to support strongly resolved relationships, or that at least some genes suggest strongly supported histories that conflict with those supported by other genes.

In general, branches that conflict between species trees are not well supported in any of the trees. For instance, in the ASTRAL-II analysis, *P. repens* is placed as the sister taxon to the entire genus

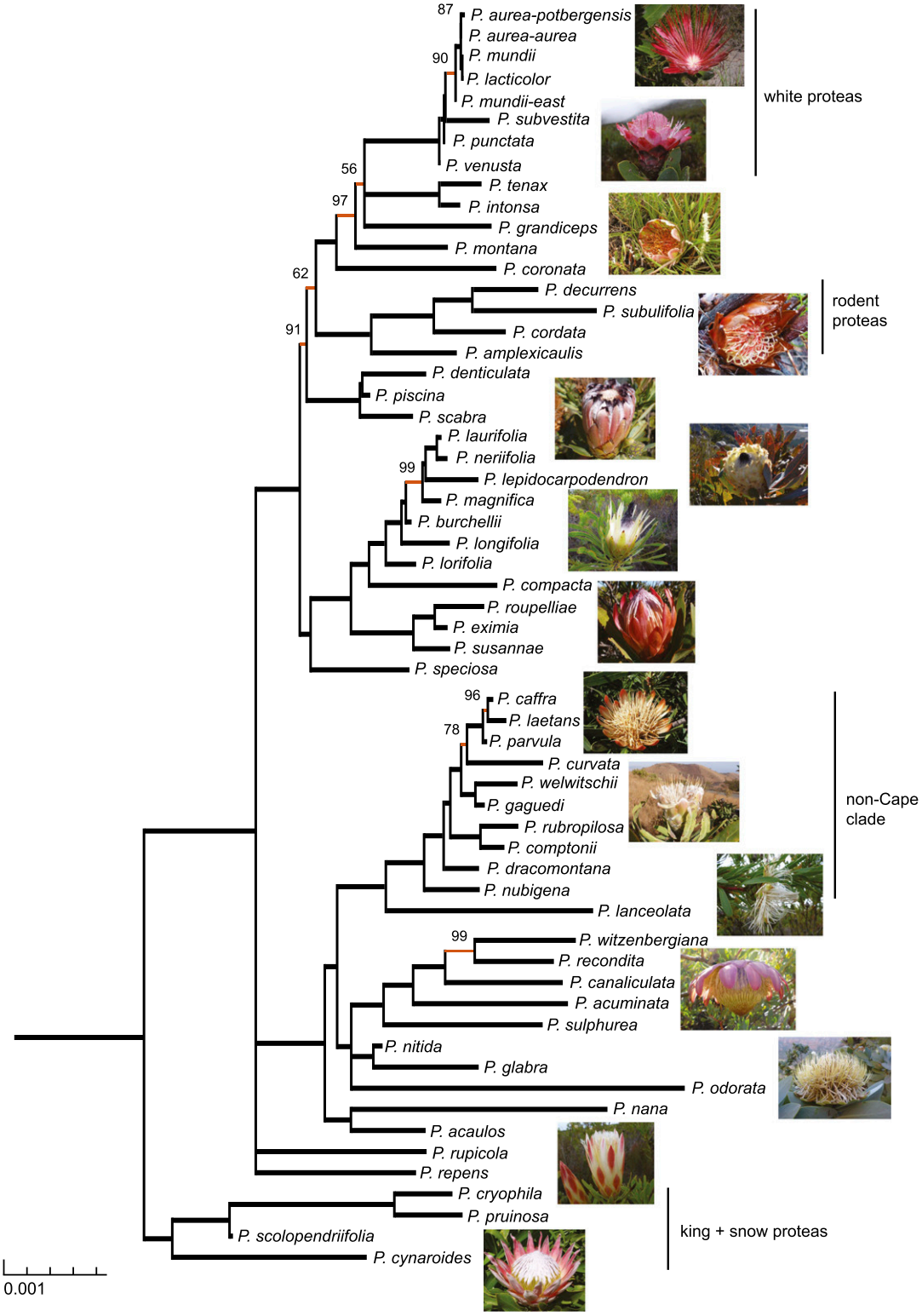

FIGURE 3 Species tree generated using ASTRAL-II. Branches with 100% bootstrap support are indicated with thick black lines; branches with less than 100% bootstrap support are orange and have support values written; branches with less than 50% bootstrap support have been collapsed. Outgroups have been removed to show details within *Protea*; for whole trees, see newick files in the supporting online material. Branch lengths correspond to the mean number of substitutions per site. Representative species are shown to demonstrate floral diversity. From top to bottom: *Protea aurea* subsp. *aurea*, *P. punctata*, *P. montana*, *P. cordata*, *P. laurifolia*, *P. magnifica*, *P. longifolia*, *P. repens*, *P. susannae*, *P. caffra*, *P. gaguedi*, *P. lanceolata*, *P. sulphurea*, *P. nitida*, *P. repens*, *P. cynaroides*. Photos credit: N. Mitchell, J. E. Carlson, and C. S. Adams.

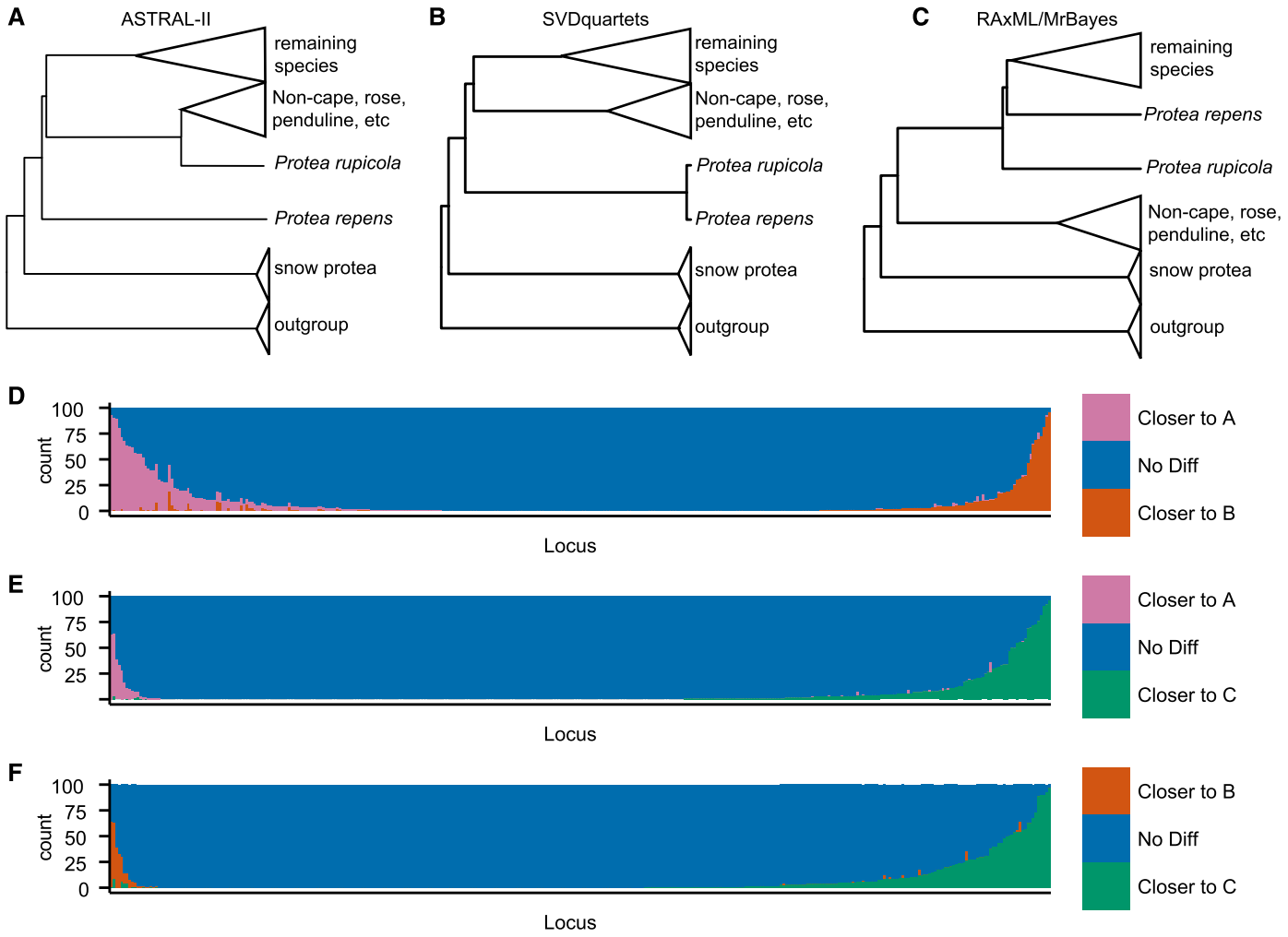

FIGURE 4 Format for ad hoc analysis of the placement of *P. rupicola* and *P. repens*, where (A) reflects the ASTRAL-II "A" topology, (B) reflects the SVDquartets "B" topology where they are sister taxa, and "C" reflects the RAxML/MrBayes grade topology. Note that this is a diagrammatic representation, and branch lengths are meaningless. (D–F) Results for pairwise comparisons for individual bootstrap replicates in gene trees, where replicates can be closer to either topology or not differ in distance. Each bar represents a different locus, and colors represent the direction of change of each replicate for (D) "A" vs. "B", (E) "A" vs. "C", and (F) "B" vs. "C".

except for the snow proteas, and *P. rupicola* is then sister to a clade nested within the remainder of the group. However, these groupings are not well supported, with bootstrap values of 49% and 42% (Fig. 3, Fig. 4A). In the SVDquartets analysis, *P. rupicola* and *P. repens* are sister species that are collectively sister to a larger clade containing all of *Protea* except for the snow proteas (Appendix S5, Fig. 4B). However, the bootstrap support underlying the sister pairing of these species is only 64%. In contrast, in the RAxML topology, these taxa form a grade within one of the major clades, with *P. rupicola* sister to a grade with *P. repens*, and *P. repens* sister to another large clade, supported with low bootstrap values of 78% and 57%. The topology from the MrBayes analysis is the same as that from RAxML, with fairly high mean posterior probabilities of 1.0 and 0.97 (Appendix S5, Fig. 4). These topologies differ from the published topologies found in Valente et al. (2010) and Schnitzler et al. (2011).

To understand the discordant placement of *P. repens* and *P. rupicola*, we performed ad hoc "alternative placement tests". Three pairwise comparisons among alternative topologies found in our trees indicate that most genes provide no information regarding the placement of these taxa. For these analyses, we use the ASTRAL-II tree as a base and then manipulated the placement of only *P. repens* and *P. rupicola* on that background. Selection of the base tree is not of extreme importance, because we are looking at differences related only to the placement of the two taxa of interest, which is manipulated by the user. Use of other species trees as backbones does not change the outcome. A gene tree having an RFadj distance closer to one topology over the other implies that that gene tree is more similar to that particular topology. In this way we can see whether genes differ strongly regarding this particular placement while controlling the rest of the tree. Here, the "A" topology reflects the topology found in the ASTRAL-II tree, "B" is the topology found in the SVDquartets tree, and "C" is the same base topology with the RAxML/MrBayes *P. repens*–*P. rupicola* placement (Fig. 4A-C). Comparing "A" and "B", we found that 22 best gene trees were closer to "A" and 25 were closer to "B", with 307 not differing (distance was the same to either topology). Similarly, when

comparing "A" and "C", 31 genes were closer to "A", and only 7 were closer to "C", and 316 did not differ. Finally, when comparing "B" and "C", 30 were closer to "B", 7 were closer to "C", and 317 did not differ. Moreover, 220 out of 354 gene trees had at least one bootstrap replicate closer to one topology or the other. Within a locus, there are bootstrap replicates closer to either topology in pairwise comparisons (Fig. 4D-F).

Internode certainty (IC, which ranges from negative one to one) values across the REDUCED 60-taxon ASTRAL-II topology were on average very low (0.076) despite overall high bootstrap support, suggesting that conflicting clades in the gene trees have as much support as the focal clade in the species tree. In fact, almost half (25/56) had zero or negative IC values, indicating that a different resolution was favored by gene tree topologies other than those found in the species tree. Even excluding negative or zero values, the average is still low (0.266). Consistent with gene trees lacking resolution, we found a positive relationship between log-transformed branch length and internode certainty (Pearson's correlation = 0.587, $t = 5$, df = 54, $P < 0.001$; Appendix S7). Relationships between branch lengths and IC for the other species trees had similar patterns: SVD had 25 zero or negative values (average IC of 0.059, 0.258 with positives only), while the identical MrBayes and RAxML trees had 26 zero or negative IC scores (average of 0.058, 0.261 positives only).

Hypothesis testing: poor support due to gene tree discrepancy—Each of the 354 individual gene trees generated in RAxML using species-level CONSENSUS data had extremely low support at nearly every branch. On average, gene trees had only 15 branches with 50% or greater support, only 5 branches with 80% or greater support, and only 2 branches with 95% or greater support (out of a possible 57 nodes in REDUCED unrooted gene trees, average support across all: 29.6%), with none of the 354 gene trees having more than 48.7% average support, and a lowest average support value of 6.5%. If we look at this in a Bayesian context, on average the majority-rule consensus gene trees from the MrBayes analysis had 14 branches with a posterior probability of 50% or higher, 10 branches with an average posterior probability of 80% or higher, and 7 branches 95% or over. The average posterior probability was only 28.3% within any individual gene tree. Bayesian analyses are not dependent on containing a reasonable chance that sites change along a branch, and thus, single sites can give strong support. It therefore seems likely that individual sites within a locus may lend some support to relationships, although there may be few sites per locus with substantial information for phylogenetic inference.

Adjusted Robinson-Foulds distances (RFadj) among the gene trees were very high, with an average of 0.912 and range of 0.667 to 1.00 across the 62,148 comparisons. In spite of these very large distances, they were more similar to each other than randomly simulated gene trees (average of 0.998, range 0.930 to 1.00), ($t = 43$, df = 375, $P < 0.001$). Gene trees simulated under the coalescent process in COAL were more similar to each other (average of 0.614, range 0.253 to 0895) than the observed best trees ($t = 67$, df = 517, $P < 0.001$) or randomly generated gene trees ($t = 96$, df = 358, $P < 0.001$), indicating that ILS alone cannot explain the discrepancies among gene trees (Appendix S8).

Gene tree-to-species tree RFadj values were fairly high for the four species trees, with averages of 0.820, 0.839, and 0.828 for ASTRAL-II, SVDquartets, and RAxML/MrBayes, respectively. Gene tree-to-species tree differences were significantly different for SVDquartets-gene and ASTRAL-gene distances ($t = 4.13$, df = 702, $P < 0.001$) and SVDquartets-gene and RAxML/MrBayes-gene ($t = 2.39$, df = 705, $P < 0.05$), though not significantly different for ASTRAL-gene and RAxML/MrBayes-gene ($t = 1.74$, df = 705, $P = 0.08$) (Fig. 2). Note that RAxML and MrBayes generated identical topologies, so distances are identical between these trees and other trees. This pattern of relatively high gene tree distance from the species trees may be due to essentially random resolution of the mostly unresolved best gene trees (given the average bootstrap support of approximately 30% for bipartitions in individual gene trees).

Hypothesis testing: poor support due to hybridization—Using the COMPLETE data set, the SplitsTree analysis (Fig. 5), identified a handful of species possibly involved in reticulation (*P. glabra*, *P. nitida*, *P. acaulos*, and *P. rupicola*) as well as some divergent individuals or sequences. Three of these (*P. scolopendriifolia* 246, *P. cordata* 42B, and *P. recondita* 58A) also had nonmonophyletic placement in the COMPLETE phylogenies, while *P. venusta* 148 was contained within the white proteas, but its two sequences were separated. Apart from these examples, the network has a distinctively tree-like topology. SplitsTree analyses for the ONEPER and COMPLETE data set showed similar patterns (Appendix S9).

DISCUSSION

Phylogenetic support—Analyses of large, multilocus data sets have improved support and enhanced resolution in many radiations and allowed for robust insights into lineage-specific hypotheses related to biogeography, trait evolution, and timing of events (Leaché et al., 2014; Tonnabel et al., 2014a; Shen et al., 2015). Similarly, the *Protea* phylogeny presented here represents a significant improvement over trees estimated from a handful of molecular markers and AFLP loci. It is difficult to compare these data sets, given nonoverlapping taxa, but for instance, in the MrBayes species tree, 59 branches (95% of the total 62) were supported with posterior probabilities over 0.95. In contrast, only 25 of 88 branches in the Schnitzler et al. (2011) analysis received posterior probabilities over 0.95 (28%) and 29 of 86 (34%) branches in Valente et al. (2010). These results are similar to those from the related genus *Leucadendron* (Proteaceae) in which adding nuclear markers led to significantly improved resolution over that achieved using ITS alone (Tonnabel et al., 2014b).

Consistency of species tree methods—There is a large body of literature dedicated to comparing and contrasting different concatenation and species tree methods with both simulated and empirical data sets (Edwards, Liu, and Pearl, 2007; Kubatko and Degnan, 2007; Gatesy and Springer, 2014; Xi et al., 2014; Tonini et al., 2015). Broadly speaking, the methods can be divided into two groups: (1) those that implicitly assume that all loci reflect the same genealogy and analyze concatenated sequences, and (2) those that allow different loci to have different genealogies and account for ILS when estimating a species tree consistent with different gene genealogies. Although there is considerable disagreement about the virtues of each approach, agreement between species tree methods and concatenation approaches suggest low levels of ILS, hybridization, or other forms of gene tree discordance. To the extent that the approaches disagree, the areas of disagreement identify clades in the tree that warrant further investigation.

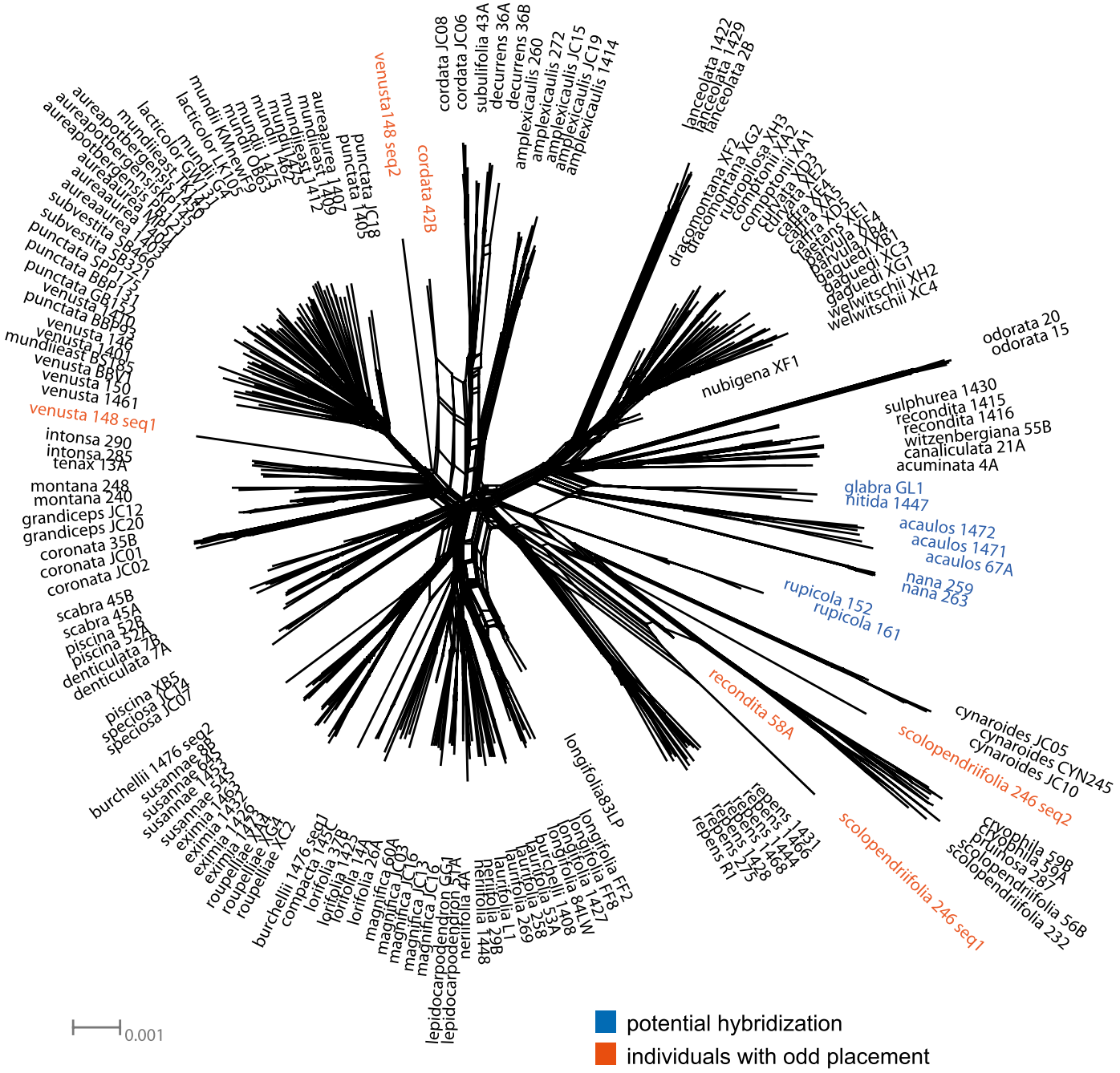

FIGURE 5 SplitsTree phylogenetic network for *Protea* samples using the COMPLETE data set. Labels are for individuals (not both alleles per individual) to save space, unless an individual's alleles were not grouped very closely together. For these, there is an additional "seq1" or "seq2" label indicating the allele. Outgroup taxa are not included to emphasize ingroup relationships. Clades with potential hybridization identified have been highlighted in blue, individuals with anomalous placement in orange. Branch lengths are in mean number of substitutions per site. Additional phylogenetic networks for the CONSENSUS and ONEPER data sets can be found in Appendix S9.

In *Protea*, concatenation and species tree methods produced similar species trees. Nonetheless, topologies generated using the two different species tree methods were more similar to each other than they were to the two concatenation-based trees. Given the rapid diversification in this genus, we expect moderate-to-high levels of incomplete lineage sorting, but we are unable to definitively declare that one method or one program works better than the others, using this data set. It could be the case that concatenation methods are able to accommodate moderate levels of ILS, and that species tree methods suffer from inaccurate gene tree inference (in the case of ASTRAL-II), which we do observe in this data set. However, the greater similarity of ASTRAL-II and SVDquartets trees to one another than to the RAxML/MrBayes tree suggests that the species tree approaches resolve possible cases of ILS in similar ways. Admittedly, this could be due to the use of the CONSENSUS data set for the concatenation analyses, and the COMPLETE data set for the

species tree methods. However, part of the appeal of programs such as SVDquartets and ASTRAL-II is the ability to account for the sampling of multiple individuals per taxon. In parts of the tree where concatenation approaches differ from the ASTRAL-II or SVDquartets-based trees, ILS or other phenomena may be invoked.

Conflicting or poorly supported clades—Within phylogenies, some branches are likely to have stronger statistical support than others. Branches that have low support may reflect short branches with few shared changes on them, incomplete lineage sorting, or hybridization. In *Protea*, branch lengths in the species trees are positively related to internode certainty, suggesting a possible lack of shared changes. However, short branches are also associated with the presence of ILS, or branches could be artificially shortened because of admixture. This finding is consistent with low support for many branches in other phylogenies associated with inferred rapid radiations, e.g., the caenophidian snakes (Pyron et al., 2014) and the diploid *Helianthus* (Stephens et al., 2015b). In addition, when we examine individual cases of species tree discrepancies using ad hoc alternative placement tests, like the placement of *P. rupicola* and *P. repens,* we find no evidence for the strongly conflicting gene trees. Although there is limited asymmetry in support for alternate topologies, the overwhelming majority of gene trees do not differ when it comes to this particular case, and there is evidence for conflicting support within individual gene trees.

Species tree: more than the sum of its parts?—When the amount of phylogenetic information contained in any one locus is quite small, the best tree at that locus is not expected to be a good estimate of species relationships. For example, many IC values were negative, meaning that the most common bipartition in the bootstrap sample was not included in the best tree. These results suggest that taking the information from best gene trees alone may not produce reliable estimates of species trees. Additionally, gene trees were topologically very different from each other and from species trees. Nonetheless, both concatenation and species tree methods produce well-resolved trees that are largely congruent. Taken together, these results suggest that while the signal at any one locus is relatively low, the signal is correlated across loci leading to a relatively strong phylogenetic signal when information from many loci is combined.

Species reciprocal monophyly—Our analyses included samples from multiple individuals for most species, and two alleles per individual, allowing us to test for reciprocal monophyly among species (Appendix S4). Overall, species formed clades, except within the white protea. This smaller radiation within the larger radiation of *Protea* is apparently quite recent, the lack of time for divergenceis reflected in short branches in the species-level phylogeny and highly intermixed groupings in the allele-level phylogeny. There is little evidence for reticulation in this group. Instead, the SplitsTree analysis suggests a star-like radiation (Fig. 5). Nonmonophyly in the white protea could affect estimates of species-level relationships in our CONSENSUS trees and could contribute to poor resolution in these analyses. Outside the white protea, a few individuals had anomalous placement, perhaps associated with high amounts of missing data (for instance, *P. recondita* 58A had only 19 loci recovered). The extent to which high amounts of missing data affect tree topologies in phylogenomics studies remains unclear, but extremes do appear to affect placement of individuals.

Hybridization—In addition to the possibility of ILS contributing to discordance between gene and species trees, there is both genetic (Prunier and Holsinger, 2010) and anecdotal (A. G. Rebelo, pers. communication) evidence of hybridization in wild populations, and breeders commonly hybridize species in cultivation for the cut flower trade (Coetzee and Littlejohn, 2007). Much of the evidence for hybridization comes from observations in the white protea subclade and the bearded sugarbushes as defined by Rebelo (2001) (e.g., *P. magnifica*, *P. longifolia*, *P. laurifolia*, *P. lepidocarpodendron*, *P. burchellii*, etc.; Appendix S1; Coetzee and Littlejohn, 2007). The phylogenetic network from SplitsTree4 does not provide a formal test for hybridization, but suggests that hybridization has not played an important role in the diversification of *Protea*. We expected to find evidence for hybridization between *P. punctata* and *P. venusta* of the white proteas, because population genetic analyses have previously detected evidence of introgression between these species (Prunier and Holsinger, 2010), yet the SplitsTree4 analysis does not detect evidence for this hybridization. Notably, there is also a lack of evidence for hybridization in the bearded sugarbushes. Areas that do seem more network-like are associated with certain individuals with divergent sequences. Apparent reticulation involving *P. recondita* 58A may be associated with large amounts of missing data. The apparent reticulation involving *P. nitida*, *P. glabra*, *P. acaulos*, and *P. rupicola* is surprising given that these species are morphologically very different. If hybridization is occurring, it may be responsible for discrepancies in the placement of *P. rupicola* in species trees estimates derived from different methods.

In collecting samples for this analysis, we avoided sampling from individuals of questionable origin in an attempt to avoid individuals of recent admixture. Thus, these results cannot be used to infer the frequency of hybridization among extant populations, only the extent of reticulation during the radiation of species in *Protea*. Additional population genetic work and more formal tests are necessary to verify the existence of recent interspecific gene flow.

Major clades of Protea—Our analyses led to a highly resolved phylogeny for *Protea*, although the traditional morphological groups defined by Rebelo (2001) are still not easily defined. Our results are, however, consistent with the strongly supported clades found in Valente et al. (2010) and Schnitzler et al. (2011). For example, the snow proteas are still very well supported as having the earliest split between their clade and all others within the genus, while the white proteas remains strongly monophyletic. Within the white protea, *P. mundii* and *P. mundii-east* did not form a clade in the SVDquartets or ASTRAL-II analyses, and grouped together with relatively low support in the RAxML and MrBayes trees, in part supporting previous work defining these geographically disjunct taxa as evolutionarily separate lineages (Prunier et al., 2014). The non-Cape clade is also highly supported, although *P. lanceolata* is consistently sister to this group. Previously, *P. sulphurea* was found to be sister to the non-Cape clade with low support (Valente et al., 2010), but it is now consistently found in a more nested position in a group outside the non-Cape species. We recover the rodent proteas as monophyletic, with high resolution within the group, despite evidence for hybridization. Many of the morphologically classified spoonbract and bearded proteas (Rebelo, 2001) form a monophyletic group as previously reported, yet several species from each group are found in different or very different parts of the tree (e.g., *P. coronata*, *P. grandiceps, P. nitida*, *P. glabra*) suggesting a possible role

for strong convergence in form. Additionally, we find strong evidence in all species tree topologies that the morphologically similar, but geographically distinct species, *P. laurifolia* and *P. neriifolia* are sister taxa, in contrast with previous findings (Valente et al., 2010; Schnitzler et al., 2011). The placement of *P. grandiceps* as sister to the white proteas is still surprising given morphology, although this relationship is not well-supported and does not differ greatly from the topologies published in previous trees (Valente et al., 2010; Schnitzler et al., 2011). The placement of *P. witzenbergiana* with *P. recondita* is also surprising, given that *P. witzenbergiana* is morphologically more similar to *P. nana*, hinting at another instance of convergence.

Incorporating sequence data from other sources—Our study included field-collected samples across South Africa, but focused on the highly diverse Cape Floristic Region in South Africa. Our samples included over half of all known *Protea* species, but it did not include some rare species, and it did not include any species outside of South Africa. To build the most complete species-level phylogeny possible, we included sequences from a different set of loci and the 39 additional taxa included in Schnitzler et al. (2011). We constrained phylogenetic estimates using these data to the 80% majority-rule consensus tree for the AUGMENTED analyses. Not unexpectedly, many branches had poor support, resulting in several "combs" where we only had sequence data from Schnitzler et al. (2011) and no anchored phylogenomics data. The trees built using different methods were, on average, more dissimilar than our nonaugmented trees, and even more different from the tree published by Schnitzler et al. (2011). These differences appear to be mostly within major clades and the placement of some clades relative to each other, likely associated with poor support and somewhat "random" resolution. These additional species do change some of our sister species groupings; for instance *P. repens* is sister to *P. aristata* in these trees, but their relative placement is still uncertain.

Although this method incorporates additional species, it is important to note that most of these relationships are still very uncertain. This is likely due to a lack of information in the Schnitzler et al. (2011) data set, but could be due to disagreement between our consensus tree and the additional data. We also do not trust branch lengths for this analysis, and therefore have not included them. These trees have limited utility, but can give a general sense of where additional species might fit. Additional statistical phylogenetic work is necessary to truly combine information from data sets with nonoverlapping sequences in a way that does not include massive amounts of missing data.

CONCLUSIONS

Using a broadly expanded phylogenomic data set, we were able to build well-resolved species-level phylogenies for the rapid radiation of *Protea*. The use of multiple approaches to tree-building allows us to identify potential areas of interest across the topology for investigating the influence of phenomena such as ILS or hybridization in the history of this group. The phylogenies generated here will allow for increased confidence in analyses of evolutionary questions in *Protea*, providing a basis for asking how diversity has been generated in this morphologically diverse, speciose, iconic plant lineage.

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APPENDIX 1.

Voucher information for specimens used in this study, accession number at CONN herbarium. *Protea acaulos* 266402, 227586; *P. acuminata* 227590; *P. amplexicaulis* 256139, 266398, 261485, *P. aurea-aurea* 1411487, 141483; *P. aurea-potbergensis* 141481, 141482*; P. burchellii* 266405; *P. caffra* 248100, 245131; *P. canaliculata* 227575; *P. compacta* 227564; *P. comptonii* 245134, 245135; *P. cordata* 227595, 230152; *P. coronata* 227581; *P. cryophila* 228689, 248060; *P. cynaroides* 227574; *P. decurrens* 230364; *P. denticulata* 230371, *P. dracomontana* 248046*; P. eximia* 266413, 266409; *P. gaguedi* 248057, 248040; *P. glabra* 255946, *P. grandiceps* 230363; *P. intonsa* 256140; *P. lacticolor* 141479, 141623, 141486; *P. laetans* 248061, *P. lanceolata* 266416, 230374; *P. laurifolia* 256127, 227477, 255937; *P. lepidocarpodendron* 255944, 230361; *P. longifolia* 227492, 266419; *P. lorifolia* 266415, 227469, 227567; *P. magnifica* 227599; *P. montana* 256141; *P. mundii* 266410, 141617; *P. mundii-east* 141619, 141618; *P. nana* 256129; *P. neriifolia* 230153, 266411, 230306; *P. nitida* 266408, *P. nubigena* 248038, *P. odorata* 256132; *P. parvula* 248054; *P. piscina* 227578, 255939; *P. pruinosa* 256133; *P. punctata* 141608, 141615, 141621, 141613*; P. recondita* 266397, 227598; *P. repens* 2566128, 266412, 266400, 255942, 266403, 266414, 266407; *P. roupelliae* 245139, 248055; *P. rubropilosa* 248041; *P. rupicola* 256137; *P scabra* 227571; *P. scolopendriifolia* 256130, 227583*; P. speciosa* 227572*; P. subvestita* 141609, 141504; *P. sulphurea* 266399; *P. susannae* 230372; *P. tenax* 230302; *P. venusta* 256134, 141500, 141606, 227596, 227695*; P. welwitschii* 245138; *P. witzenbergiana* 227598, *Faurea rochetiana*; *F. saligna*; *Serruria adscendens*; *S. furcellata*; *S. phylicoides* 266418; *S. trilopha*.

Author Contributions

N. Mitchell designed the framework for this study, assisted in the collection of samples, extracted DNA, built all phylogenies, ran analyses, and wrote this manuscript. P.O. Lewis added crucial advice and suggestions for phylogeny building. A.R. Lemmon and E. Moriarty-Lemmon oversaw library preparation and carried out initial bioinformatics processing. K.E. Holsinger thought up the alternative placement test and assisted in the organization and revision of this manuscript.

Online supplementary material for Chapter 2 may be found at:

American Journal of Botany: http://www.amjbot.org/content/104/1/102/suppl/DC1

Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.vj32s

Appendix S1: Sample information for individuals and species used in this phylogenetic study.
DOI: 10.3732/ajb.1600227.s1
http://www.amjbot.org/content/suppl/2017/01/19/ajb.1600227.DC1/Mitchell_AppS4.pdf

Appendix S2: Locus information for all 498 nuclear loci used in this study.
DOI: 10.3732/ajb.1600227.s2
http://www.amjbot.org/content/suppl/2017/01/19/ajb.1600227.DC1/Mitchell_AppS2.docx

Appendix S3: Comparisons among the CONSENSUS, COMPLETE, and ONEPER species trees.
DOI: 10.3732/ajb.1600227.s3
http://www.amjbot.org/content/suppl/2017/01/19/ajb.1600227.DC1/Mitchell_AppS3.docx

Appendix S4: Assessment of species monophyly.
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http://www.amjbot.org/content/suppl/2017/01/19/ajb.1600227.DC1/Mitchell_AppS4.pdf

Appendix S5: Species trees generated using the COMPLETE dataset for (A) SVDquartets and the CONSENSUS dataset for (B) ASTRAL-II (C) SVDquartets and (D) RAxML/MrBayes.
DOI: 10.3732/ajb.1600227.s5
http://www.amjbot.org/content/suppl/2017/01/19/ajb.1600227.DC1/Mitchell_AppS5.docx

Appendix S6: Phylogenies incorporating sequences and species from Schnitzler et al. (2011) (AUGMENTED dataset), using the 80% SVDquartets (A) ASTRAL-II (B) SVDquartets or (C) RAxML/MrBayes species tree topologies as a backbone.
DOI: 10.3732/ajb.1600227.s6
http://www.amjbot.org/content/suppl/2017/01/19/ajb.1600227.DC1/Mitchell_AppS6.docx

Appendix S7: Scatterplots with smoothed lines for the relationships between log-transformed branch lengths and internode certainty for ingroup nodes for the (A) ASTRAL-II, (B) SVDquartets, and (C) RAxML/MrBayes species trees.

DOI: 10.3732/ajb.1600227.s7
http://www.amjbot.org/content/suppl/2017/01/19/ajb.1600227.DC1/Mitchell_AppS7.docx

Appendix S8: Violin plots for adjusted Robinson-Foulds distances among observed gene trees, randomly generated trees, and among trees simulated from the ASTRAL-II tree using COAL.
DOI: 10.3732/ajb.1600227.s8
http://www.amjbot.org/content/suppl/2017/01/19/ajb.1600227.DC1/Mitchell_AppS8.docx

Appendix S9: SplitsTree diagrams for (A) CONSENSUS sequences and the (B) ONEPER sequences.
DOI: 10.3732/ajb.1600227.s9
http://www.amjbot.org/content/suppl/2017/01/19/ajb.1600227.DC1/Mitchell_AppS9.docx

Chapter 3:

Trait-environment co-evolution contributes to adaptive differentiation in *Protea*

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Abstract

Rapid evolutionary radiations are responsible for much of Earth's diversity, yet the causes of these radiations are often elusive. Determining the relative roles of adaptation and geographic isolation in diversification is vital to understanding the causes of any radiation. Trait-environment relationships suggest that traits play an important role in contemporary ecology, either via *in situ* adaptation to local environments or via environmental filtering of traits that are already differentiated. We examine contemporary and evolutionary associations, divergence order tests, and models of evolution on a strongly supported phylogeny in the iconic plant genus *Protea* to identify co-evolution of traits and the environment species occupy. Results indicate that trait diversification in *Protea* has been broadly adaptive, with co-evolution of plant size with temperature and leaf investment with rainfall. Contemporary trait-environment relationships are consistent with many co-evolutionary associations, even though some of these associations are inconsistent with global patterns on a broader phylogenetic scale. Neither *in situ* adaptation nor environmental filtering is predominantly responsible for contemporary trait-environment associations, but there is limited evidence for each in a few traits.

This work is in revision for the journal Evolution.

Introduction

Evolutionary radiations are responsible for much of the diversity of life on Earth. They are often characterized by rapid diversification of lineages into new species and morphological forms (Schluter 2000). The extent to which diversification is driven by adaptive processes and natural selection often remains unclear (Givnish 1997), since radiations can also be the byproduct of divergence via geographic isolation associated with stochastic or neutral processes (non-adaptive radiations, Kozak et al. 2006, Rundell and Price 2009). Tracing the simultaneous evolution of individual traits and environments is one way to assess the role that adaptation played in generating diversity. If adaptation is important in trait diversification, then evolutionary changes in traits will be associated with changes in the habitat or environment that species occupy. In particular, changes in functional phenotypic traits, those with presumed effects on survival, growth, and reproduction in the context of the abiotic environment (Violle et al. 2007), should be associated with changes in some aspect of the environmental or climatic niche, though changes in biotic associations may also play a role. Trait-environment associations across the branches of a phylogeny are evidence that broad-sense adaptation plays a role in trait diversification. There is also a wealth of evidence for integrated trait evolution in plants (e.g. the worldwide leaf economics spectrum, Wright et al. (2004)), so patterns of covariation in traits and environment also need to be identified.

Trait-environment associations can be observed at many spatial and temporal scales. For example, statistical associations between field-measured traits and environmental parameters provide evidence that contemporary trait differences are associated with important physiological and ecological functions both among and within distantly related genera in South Africa (Mitchell et al., 2015). By themselves, such associations do not provide evidence that differentiation in those functions played an important role in evolutionary diversification among species. Evidence for the adaptive nature of trait-environment associations is strengthened by analyses at both contemporary and evolutionary time scales. Specifically, assessing co-evolutionary associations across a phylogeny requires the use of comparative

methods (e.g., phylogenetically independent contrasts (Felsenstein 1985) or phylogenetic generalized least squares (Martins and Hansen 1997)). These approaches provide measures of trait-trait or trait-environment associations while controlling for phylogeny, i.e., 'evolutionary associations'. Contemporary associations, in contrast, are correlations measured without taking phylogeny into account, and their underlying causes are harder to interpret, because they can be skewed by increased speciation rates within some lineages, phylogenetic constraints on trait evolution, or other factors. If contemporary and evolutionary associations are consistent, however, then the evidence for adaptive (*sensu lato*) mechanisms shaping these patterns is stronger.

Diversification may also occur whenever there is geographic, ecological, or evolutionary opportunity (Simpson 1955; Glor 2010; Simões et al.). Geographic opportunity may be provided by nothing more than geographic isolation. Ecological opportunity may be associated with entering a new "adaptive zone", defined as the suite of environmental conditions that determines the types of adaptations of a lineage (Simpson 1955), followed by adaptation within the new environment. Evolutionary opportunity may be associated with a key innovation that allows a lineage to enter new environments (Mayr 1963). Abiotic filters may produce in trait-environment associations by preventing or limiting establishment, survival, or reproduction of individuals or species lacking traits suitable for a particular environment (reviewed in Kraft et al. 2015). In any of these scenarios, a pre-existing trait may *allow* species to occupy a new environment (environmental filtering, Fig. 1A). Alternatively a trait may evolve *after* a lineage enters the new environment (environmental adaptation, Fig. 1B) (Simpson 1944). Environmental filtering and environmental adaptation are extreme ends of a spectrum, and it is likely that both occur when phenotypic traits have strong relationships to performance that vary with the environment (Fig. 1C).

Trait divergence in an adaptive radiation may be associated with either environmental filtering or environmental adaptation. In one scenario, adaptive radiation follows a sequence of stages where organisms first diverge in their climatic-niche at the macrohabitat scale followed by divergence at the microhabitat scale and in locally adapted traits (perhaps associated with biotic interactions) that enhance

survival and reproduction only at the latest stages of the radiation (Gavrilets and Losos 2009). Under this scenario, differences in the climatic niche or habitat arise before differences in phenotypic traits (e.g., the habitat-first model of Diamond (1986)),, traits evolve to fit new environments that species occupy, and trait-environment associations are the result of *in situ* adaptation (Fig. 1B, C). For example, climate niche parameters appear to be more closely associated with species richness and diversification than phenotypic differences are in plethodontid salamanders (Kozak and Wiens 2016). In another scenario, traits may diverge as a result of geographic isolation, and environmental filtering accounts for contemporary trait-environment associations. For example, Ackerly et al. (2006) found that differences in a functional trait (LMA, leaf mass per area) in the plant genus *Ceanothus* evolved before differences in climatic niche parameters (rainfall and temperature) were apparent, a pattern consistent with environmental filtering rather than environmental adaptation (Fig 1A, C). Other examples of environmental filtering are found in *Anolis* lizards (Glor et al. 2003) and *Phylloscopus* warblers (Richman 1996).

Furthermore, comparisons of models of evolution can help to determine whether phylogenetic signal in traits could be the result of phylogenetic niche conservatism. If both are relatively conserved, then the environment may have played a role in trait evolution. If one or the other is not conserved, local or contemporary processes may be more important.

Existing methods for analyzing trait-by-trait or trait-by-environment associations at evolutionary timescales suffer from two important limitations. Basic methods sometimes assume that traits are uniform within species and they ignore uncertainty in phylogenetic estimates (but see Huelsenbeck et al. (2000). These limitations may be especially important in rapid radiations, where soft polytomies are abundant and species relationships may be uncertain. The use of Bayesian posterior tree samples or bootstrap replicates can account for some phylogenetic uncertainty (Huelsenbeck and Rannala 2003), and very recent methods have begun to incorporate this uncertainty into estimates of correlated trait evolution (Caetano and Harmon 2017), but the role of intraspecific trait variation has often been neglected. The magnitude of intraspecific trait variation is often quite large, especially in some often-used plant functional traits (Auger and Shipley 2013; Donovan et al. 2014; Carlson et al. 2015). This variation may have large

impacts on comparative studies (Garamszegi and Møller 2010), motivating the modeling of trait variances as well as means in evolutionary studies (Kostikova et al. 2016). These analyses, however, are difficult to carry out on large datasets, including many species..

We ask whether the evolution of traits and environment reflect adaptive evolution in the radiation of the plant genus *Protea*, a lineage with its center of diversity in the Cape Floristic Region of South Africa, a biodiversity hotspot. More specifically, we ask:

1a) Have traits and the environment evolved together?

1b) Do contemporary and evolutionary patterns of trait-environment associations match?

1c) Do best models of evolution indicate that similarities in traits between descendant and ancestral species are driven by similarities in environment?

2) Are patterns of integrated evolution consistent with patterns observed in global datasets?

3) In trait-environment pairs for which we detect coevolution, can we distinguish environmental adaptation (environments diverge first) from environmental filtering (traits diverge first)?

The answers to these questions allow us to identify trait-environment associations and place them along the continuum from environmental filtering to environmental adaptation (Fig. 1C). In answering these questions, we also address how phylogenetic uncertainty and variation in both traits and environmental niche values affect our conclusions.

Methods

All analyses were carried out in R v3.3.1 (R Core Team 2016) and largescale analyses were carried out on the Computational Biology Core Facility of the University of Connecticut.

STUDY SYSTEM

The genus *Protea* L. (Proteaceae) is a diverse group with 112 known evergreen plant species displaying diversity in growth form (ranging from sub-shrubs to shrubs and small trees), leaf shape and

size, and inflorescence architecture (Rebelo 2001; Valente et al. 2010). The age of the group is uncertain, but best estimates place the crown age at 5-18 my (Sauquet et al. 2009). The genus has its center of diversity in the Cape Floristic Region (CFR) of South Africa (Valente et al. 2010), a biodiversity hotspot characterized by high levels of species diversity (over 9000 plant species) and endemism (about 70%, Goldblatt and Manning 2002) in addition to being particularly threatened by human impacts (Myers et al. 2000). *Protea* is one of the dominant members of the fynbos community, although its range extends into northern and eastern portions of South Africa, Lesotho, Kenya, and central Africa (Rourke 1980; Rebelo 2001; Valente et al. 2010). Previous studies have documented contemporary associations between morphological traits and the environment across the genus (Mitchell et al. 2015), within smaller clades (Carlson et al. 2011; Prunier et al. 2012), and within species (Carlson et al. 2015).

The extraordinary plant diversity in South Africa has been attributed to several different factors: the topographical complexity of multiple mountain ranges and "sky islands", sharp changes in soil types, soils that are extremely low in nutrients, steep gradients in temperature and in rainfall amount and seasonality, and the onset of the present day climate dated at the Miocene-Pliocene boundary some 10 million years ago (Linder 2003; Verboom et al. 2009; Verboom et al. 2015). This diversity is largely accounted for by high species diversity in just 33 evolutionary radiations (Linder 2003; Linder and Hardy 2004; Schnitzler et al. 2011). The extent to which climatic and environmental heterogeneity has driven speciation and radiation throughout the region remains to be determined.

TRAIT MEASUREMENTS

We measured a suite of traits on plants from 58 different *Protea* species in the field from 2011-2013, including leaf and whole plant traits. We incorporated additional traits measured by Carson et al. (2011) from 2008-2009, resulting in 133 species x site combinations that covered most of the range of *Protea* (Fig. 2, average # of observations per species = 26, range = 1 – 203. There were 1520 observations, of which less than 10 percent are complete, by design (See Table S1 for full data). For most populations (species x site combinations), we sampled eight plants for trait measurements, including

height and canopy area (estimated from measured orthogonal dimensions of the plant using the formula for an ellipse) and sampled one of the most recently fully expanded leaves per plant. For shrub-like species, we also harvested wood samples from the previous year's growth for two plants per population. We measured leaf fresh weights and scanned leaves for analysis of length, width, and area in ImageJ (National Institutes of Health, Bethesda, MD, USA). Leaves were then dried and re-weighed for dry weights. For one to two leaves per population, we made stomatal peels on the adaxial side using clear nail varnish and tape that were later analyzed under a light microscope to estimate stomatal size and density. Four leaves per population were analyzed for carbon and nitrogen isotopes at the Stable Light Isotope Laboratory in the Archaeology Department at the University of Cape Town. Wood density was estimated using a water displacement method as dry mass / wet volume (Cornelissen et al. 2003). We combined these data with similar data reported for the white *Protea* clade in Carlson et al. (2011). Final traits used in this analysis include the following: plant height (cm), plant canopy area (cm^2), leaf mass per area (lma, $g \cdot cm^{-2}$; dry leaf mass / fresh leaf area), leaf fresh water content (fwc; [leaf fresh weight – leaf dry weight / leaf dry weight]), leaf length to width ratio (lwr; leaf length / leaf width), leaf area (cm^2), stomatal density (sd, $stomates \cdot cm^{-2}$), leaf nitrogen per unit mass (nmass; $mg \cdot g^{-1}$), leaf ^{13}C / ^{12}C ratio ($\delta^{13}C$, d13c), leaf carbon to nitrogen ratio (cnratio), and wood density (wood, $g \cdot cm^{-3}$), Table 1. We natural-log-transformed all traits except for d13c prior to analysis.

Instead of using species means to characterize traits in comparative analyses, we used Bayesian models to estimate the distribution of trait values within species. Specifically, we used the stan_glmer (or stan_glm if only one population) function in the R package "rstanarm" (Gabry and Goodrich 2016) to model values for each log-transformed trait and each species using an informative prior from a normal distribution (with a mean of the actual trait mean, and standard deviation of the actual standard deviation in trait values, used to compare changes across traits) and a site random effect. We ran each model for 5000 iterations saved 10,000 samples from each posterior distribution. We calculated mean values and the 95% highest posterior density (HPD) intervals (using R/coda) and randomly sampled 100 values per trait

per species for use in analyses of trait evolution. See Fig. S1 for density plots of 100 randomly selected samples per species per phenotypic trait.

ENVIRONMENTAL NICHE CHARACTERIZATION

We used Maximum Entropy Modeling (MaxEnt, Phillips and Dudik 2008) to characterize the niche for all 58 *Protea* species in our dataset. Latitude and longitude occurrence data for each species were extracted from the Protea Atlas database <http://www.proteaatlas.org.za/>. The occurrence data included 94,715 individual geo-referenced records across our species. Environmental variables for each georeferenced point were extracted from the South African Atlas of Agrohydrology and Climatology layers (Schulze et al. 2007) at the resolution of 1 by 1 minute, or 1.55 by 1.85 km. Sites were grouped together if they were in the same grid cell, and all species observed in that grid cell were recorded. We retained ten variables that capture climatic gradients in the CFR and were used previously in the literature (Table 1): mean annual temperature (mat), average daily minimum temperature in July (tmin), average daily maximum temperature in January (tmax), elevation (elev), mean annual precipitation (map), inter-annual rainfall variability, measured as the coefficient of variation of mean annual rainfall across years (rflcv), temperature variability measured as maximum – minimum temperature at a site (tvar), mean annual potential evapotranspiration (pet), and two more direct measures of drought: the number of days receiving greater than 2mm of rain in the three driest months, hence lower values reflect more drought (rfl2mm), and mean summer rainfall, December – February (summer_rain). All analyses were performed using MaxEnt in R through the package "dismo" (Hijmans et al. 2013). For each species in the dataset, we used 90% of the data for training and left 10% for testing. Random pseudo-absences were taken from the extent of South Africa, because species of *Protea* are found in most South African biomes.

We used the raw probabilities generated from the MaxEnt models to generate histograms for each species and square-root transformed environmental variables (except for tmin, which was left untransformed) using custom scripts from S.D. Smith (see Evans et al. 2009). These give a distribution of the predicted occupancy profile for each species in each climate variable independent of the other

variables. From each of these distributions we randomly sampled 10,000 observations, then calculated the 95% HPD intervals for each variable and each species in the R package "coda" (Plummer et al. 2006). From these 9,500 samples, we calculated means per variable per species and also randomly selected 100 observations for use in downstream analyses. Fig. S1 has density plots of 100 randomly selected samples per species per niche trait.

ACCOUNTING FOR UNCERTAINTY IN PHYLOGENY AND TRAITS

It is often difficult to estimate phylogenetic relationships in rapid radiations (Knowles and Chan 2008). In earlier work we used an anchored phylogenomics approach (Lemmon et al. 2012) to sequence almost 500 nuclear genes conserved across all angiosperms (Buddenhagen et al., In Review) and built a robust and highly resolved phylogeny for 59 *Protea* species (Mitchell et al. 2017; Buddenhagen et al. In Review). To ensure that our results are robust in the face of both phylogenetic and trait/environment uncertainty, we compared results of analyses using the "best" tree from the program ASTRAL-II (Fig. 3) (Mirarab and Warnow 2015) or 100 bootstrap replicates from the ASTRAL-II analysis of Mitchell et al. (2017) with either the posterior mean of the trait/environment distribution or 100 random samples from the HPD distribution. We thus have a two-by-two table comparing one measure (mean trait on the best tree), 100 measures (mean trait on 100 bootstrap trees; 100 observations of traits on best tree), or 10,000 measures (100 observations of traits on 100 bootstrap trees). From here forward, we refer to datasets as number of trait/environment observations × number of bootstrap trees, organized in order of increasing effort to account for uncertainty (1×1, 1×100, 100×1, 100×100). We sort the outputs from 100×100 analyses and select the 2.5%, 50%, and 97.5% values to compare with the lower, middle, and upper bounds with the 1×1 analyses.

CORRELATED EVOLUTION BETWEEN TRAITS AND ENVIRONMENT

We tested for correlated trait and environment evolution using the program BayesTraits (Pagel and Meade 2007) through R using the wrapper package "btw" (Griffin 2015). BayesTraits analyzes

continuous traits using a PGLS framework under the assumption of Brownian Motion. For each set of trees and morphological traits/environmental variables, we evaluated a model using the continuous function under MCMC settings, estimating the log marginal likelihood using the stepping stone method (SS, Xie et al. 2010) with 100 stones and 1,000 iterations per stone (increasing the number of stones to 200 or iterations per stone to 10,000 had little effect on estimates, data not shown). We estimated two times the log Bayes Factor (abbreviated as logBF) for the dependent model (allowing correlation between variables) against the independence model (which fixes all correlations to be zero) using the formula 2*logBF = 2(SSdep - SSindep), where SSdep and SSindep are estimated log marginal likelihoods for the dependent and independent models, respectively. A logBF > 2 was interpreted as having weak support, logBF >5 as having moderate support, and logBF > 10 as having strong support. This analysis was performed on all 110 trait-environment combinations for the 1×1 and 100×100 datasets.

ACCOUNTING FOR EVOLUTION

We investigated the joint evolutionary history of traits and environment above using BayesTraits, but the investigation of contemporary patterns can potentially provide additional information. If adaptation drives trait-environment relationships, we expect contemporary and evolutionary patterns to be similar, whereas if environmental filtering is the driver, contemporary and evolutionary correlations are likely to differ. We estimated the correlation coefficients for all pairwise comparisons (trait-by-trait, trait-by-environment, and environment-by-environment) to determine the strength and direction of contemporary relationships using the two-sided implementation of the cor.test() function in R. We then compared the correlation coefficients between the contemporary and evolutionary scales.

EVOLUTIONARY MODELS

A default assumption for trait evolution is that ancestor and descendant species will resemble each other. If phenotypic trait diversification in a radiation is driven by the environment, we should expect not only resemblance in traits, but also some degree of niche conservatism, where descendant

species occupy environments similar to those of their ancestors (Peterson 1999). If, however, phylogenetic similarity in traits is not driven by the environment, we may find a lack of niche conservatism.

To determine the evolutionary model that best fits each trait or environmental niche, we used the fitContinuous function in the R package "geiger" (Harmon et al. 2008) to compare the fit under Brownian motion (BM), Ornstein-Uhlenbeck (OU), and white noise models. Model fit was evaluated using AIC and AICc scores. The "best" model was saved for each trait and environmental variable across all four datasets (1×1, 1×100, 100×1, and 100×100).

CORRELATED EVOLUTION AMONG TRAITS AND AMONG ENVIRONMENTS

A priori we expect correlations among traits and among environmental variables in our dataset. We estimated coefficients at the evolutionary scale using BayesTraits as above for the 55 trait-trait and 45 environment-environment comparisons. We built separate distance-based dendrograms for traits and environmental variables using the correlation matrices from the 1×1 BayesTraits analysis to identify clusters for later analysis. To visualize these trait-trait and environment-environment patterns of correlation, we built "schlichtograms" (Schlichting and Pigliucci 1998) based on correlation coefficients and support values.

TIMING OF EVOLUTION

If environmental adaptation is responsible for trait-environment associations, then environmental divergence among species should precede trait divergence. If on the other hand environmental filtering is responsible for these associations, then trait divergence should precede environmental divergence. We used the divergence order test (Ackerly et al. 2006) to assess the timing of differentiation between traits and environmental niche characteristics. This test incorporates ancestral state estimation for continuous traits using the program ANCML (Schluter 1997) and R scripts from Ackerly (2006) to calculate the DOT statistic based on the age of divergence (weighted by unstandardized contrast values) of the two traits (*D*

= *Wi* – *Wj*, where *Wi* = weighted age of the morphological trait and *Wj* = weighted age of the environmental variable) and calculate a p-value associated with D based on 200 bootstrap replicates (see Ackerly 2006 for more details). A negative value of D indicates that the environmental trait diverged earlier, while a positive D means the morphological trait diverged earlier. P-values were calculated based on two-tailed T-tests on the bootstrap replicates for individual pairwise comparisons.

SUPPLEMENTAL ANALYSES

We performed two additional sets of analyses to ensure that our results are insensitive to important modeling choices. First, to assess the influence of log-transforming trait and environmental data we performed all analyses on the 1×1 datasets using untransformed data and found results qualitatively similar to those we obtained using log-transformed data. Second, to assess the influence of the method of phylogenetic inference we used, we also performed all analyses on the 1×1 datasets using the best trees identified using SVDquartets (Chifman and Kubatko 2014) and RAxML (Stamatakis 2014) and found results qualitatively similar to those we obtained using ASTRAL-II.

Results

TRAIT-ENVIRONMENT CORRELATED EVOLUTION

We detected coevolution between morphological and environmental niche traits in only a relatively small number of cases in two main clusters of strongly supported evolutionary associations. These include (1) plant size (leafarea, height, wood density) and its positive association with temperature (mat, tmax, tmin) and negative relationships with rflcv and elev and (2) leaf composition (fwc, d13c, lwr, nmass, cnratio, lma) with precipitation (summer_rain, map), where higher investment leaves are found in drier areas (Fig. 4). In addition, stomatal density has a strongly supported positive association with elevation. In terms of individual pairwise associations, estimated correlation coefficients for the BayesTraits analyses ranged from -0.513 to 0.627 in the 1×1 analyses, but only eight of 110 were very

strongly supported. Nine additional correlations were strongly supported, 17 were weakly supported, and the remaining 76 lacked substantial support. The most strongly supported evolutionary correlations were between plant size (height) and variables related to elevation or temperature (mat, elevation, and tmin), where taller plant are found in warmer areas (Fig. 4).

CONTEMPORARY VS EVOLUTIONARY TRAIT-ENVIRONMENT ASSOCIATIONS

To compare contemporary and evolutionary associations across trait-trait, environment-environment, and trait-environment comparisons, we estimated pairwise correlations among contemporary values and compared them with the BayesTraits correlations. These associations are largely similar, there is a positive association between contemporary and evolutionary correlation coefficients (Kendall's tau = 0.818, $p < 0.001$, Fig. 5). Furthermore, in the fifty-nine (out of 210) correlations that were strongly supported in both analyses (logBF > 10, $p < 0.01$), all had the same sign. In only four cases did results strongly supported in one of the analyses have the opposite sign in the other.

MODELS OF EVOLUTION

Morphological and environmental niche traits appear to evolve in largely similar ways when analyses are based on the "best" tree and on trait or environment means for each species. All analyses of morphological traits for the 1×1 analysis (best tree and mean trait value) favored the OU model that indicates evolution around an optimum, or phylogenetic inertia in traits with limits on trait variance (Fig. 6). OU models were also consistently supported in analyses incorporating uncertainty (the 1×100, 100×1, and 100×100 analyses), although some replicates included support for BM or white noise models (particularly in canopy area, leaf area, and wood density). Six of 11 traits had replicates with support for BM, though in a small percentage of cases (the highest was in leaf area where 31% of observations in the 100×100 analysis supported BM). Thus, the morphological traits of descendants are largely similar to those of their ancestors, and the range of morphological trait variation in the genus is somewhat constrained.

Analyses of environmental niche traits also favored OU models for many environmental variables (mat, map, elev, rfl2mm, rflcv, summer_rain, and tmax) when analyses are based on the "best' tree and on species' environment means. When uncertainty is accounted for, however, they all favored a white noise model except for the summer_rain variable, for which an OU model was favored. Thus, the environmental niche traits of descendants are largely unrelated to those of their ancestors.

CORRELATED EVOLUTION ALONG AXES OF VARIATION

BayesTraits analyses reveal a wide range of pairwise correlations among morphological traits and among environmental variables (Fig. 7A and C). We identified three axes of morphological traits with strong patterns of covariation based on BayesTraits correlations: (1) general size (leafarea, height, wood density), (2) leaf composition (cnratio and lma; d13c, lwr, nmass), and (3) canopy area and fwc. Stomatal density (sd) appears to evolve somewhat independently of the other traits (Fig. 7A). Among morphological traits, estimated correlations ranged from -0.994 to 0.532 in the 1×1 analyses. Eight of the 55 correlations had very strong support (logBF > 10), five had strong support (10 > logBF > 5), and 16 had weak support (5 > logBF > 2). The remaining 26 had very weak support (logBF < 2; Fig. 7A).

Among environmental variables, we identified three major suites of environmental variables with strong patterns of covariation, likely due to measuring slightly different aspects of the same environment: (1) temperature (mat, tmin, and tmax), (2) rainfall (summer_rain, map, rfl2mm), and (3) a group combining temperature, rainfall, and variability (pet, rflcv, elev, tvar) (Fig. 7C). There is also an association between the temperature and rainfall axes of variation. Estimated BayesTraits pairwise correlations for environmental variables ranged from -0.905 to 0.865 in the 1×1 analyses. Twenty-one of the 45 pairwise correlations were very strongly supported, four were strongly supported, six were weakly supported, and 14 lacked support (Fig 7C). The strongest correlations were those between elevation and tmin (corr = -0.905, logBF = 94.5) and map and rfl2mm (corr = 0.865, logBF = 78.8).

Incorporating trait uncertainty in the BayesTraits analyses (100×100 dataset) resulted in qualitatively similar outcomes (Fig. 8A). The median values of the correlations for the 100×100 dataset

were similar to the values from 1×1 analyses, although they tended to be less extreme. Notably, the upper and lower (97.5^{th} and 2.5^{th} percentiles) indicate that point estimates are very imprecise. In contrast, using other species trees has a minor influence on point estimates using other species trees, and the interval of the estimates is fairly narrow, indicating that phylogenetic uncertainty is not contributing heavily to differences in these estimates (Fig. 8B).

TIMING OF DIVERSIFICATION

Results from the divergence order tests fail to provide convincing evidence that specific traits led to occupation of new environments (i.e. filtering) and they fail to provide convincing evidence that the occupation of new environments led to the evolution of new trait values (i.e. adaptation). Only 15 out of 110 tests were statistically significant ($p < 0.05$) in the 1×1 analyses (Table 2), and none were significant after correcting for multiple comparisons with a false discovery rate correction (Benjamini and Hochberg, 1995). Nonetheless, six of those 15 differences arise because species are estimated to have diverged in wood density before occupying new environmental niches, and four are cases where stomatal density diversified after environmental niche. After incorporating uncertainty using the 100×100 analyses, there were no significant cases where environment diverged first. All ten significant estimates of D are instances of trait-first divergence, and seven are related to wood density. Divergence differences calculated using 100×100 analyses and different species trees are consistent with those from our ASTRAL-II tree (Fig. S2). Thus, there is at best suggestive evidence for environmental filtering for wood density and environmental adaptation for stomatal density, and there is no convincing evidence that either process dominates in *Protea*.

Discussion

CORRELATED TRAIT-ENVIRONMENT EVOLUTION INDICATIVE OF ADAPTIVE RADIATION

Our results provide several examples where morphological traits have a shared evolutionary history with environmental niche traits, suggesting that differentiation in these morphological traits was adaptive. These overall patterns include the positive association between plant size and temperature, and the negative association between leaf investment and rainfall amount. It is a common pattern that plants tend to be taller and have larger leaves with dense wood in warm areas and to be shorter with smaller leaves and less dense wood at high elevations see (Kdimer 1999). We found similar contemporary trait-environment relationships in this and previous studies (Carlson et al. 2011; Mitchell et al. 2015). Based on the positive relationship between evolutionary (BayesTraits) and contemporary associations, we show that these specific relationships reflect a shared evolutionary history. Specifically, plants in areas with low rainfall invest more in their physical construction, with higher values of LMA and wood density, higher C:N ratios, lower nmass, and lower leaf water contents. These results are consistent with the hypothesis that higher values of LMA are associated with greater investments in resource conservation (Wright et al. 2004). Similar associations between the evolution of morphological traits and shifts in environmental niche have also been seen in *Pelargonium* in the CFR (Jones et al. 2013), but the trait-environment associations found in *Protea* and *Pelargonium* can differ (Mitchell et al. 2015). For example, larger plants are associated with more drought in *Pelargonium*, while the opposite is true in *Protea*. Similarly, the positive association between leaf mass per area and temperature in our data is opposite from the relationship found in global databases (Wright et al. 2004), and within the CFR, *Leucadendron* shows no detectable relationship between temperature and leaf area (Thuiller et al. 2004).

The congruence between contemporary and evolutionary analyses suggests that broad sense adaptation played an important role in trait diversification in *Protea* and in generating contemporary phenotype-environment associations. Seventy percent of pairwise trait-environment correlations had the same sign in contemporary and evolutionary analyses. Only three of the correlations that differed were strongly supported; all of them were contemporary correlations of traits with potential evapotranspiration. This result could indicate that aridity places strong limits on lma, fwc, and $\delta^{13}C$.

MODELS OF TRAIT AND ENVIRONMENTAL NICHE EVOLUTION

Morphological traits are evolutionarily conserved in *Protea*, but this does not appear to be the result of phylogenetic niche conservatism. An Ornstein-Uhlenbeck model consistently provides the best fit to data from morphological traits, indicating that the morphological traits of descendants not only resemble those of their ancestors but also that the range of variation within the genus is constrained over time (Cooper et al. 2010). In contrast, once variation in the environment occupied by a species is accounted for, a white noise model provides the best fit to the data for environmental niche traits indicating that the environment occupied by descendants seems to be largely unrelated to that of its ancestors. Alternatively, Skeels and Cardillo (2017) found evidence for multiple-optima OU models demonstrating phylogenetic niche conservatism in *Protea* with differences between hotspot and non-hotspot clades. The variety of models tested and degree of uncertainty incorporated warrants further investigation into the degree of niche conservatism in this group. At best, phylogenetic niche conservatism may be characteristic of *Protea* only within certain subclades.

One environmental variable is evolutionarily conserved: the summer rainfall variable. This finding probably reflects the invasion of eastern South Africa by a single, "non-Cape" clade and the dramatic contrast in summer rainfall between the CFR and eastern South Africa. Similarly, elevated species diversification rates are associated with differentiation along this summer rainfall gradient in *Pelargonium*, though in the opposite direction, from east to west (Martínez-Cabrera and Peres-Neto 2013). However, overall in *Pelargonium*, there is overall a lack of environmental niche conservatism (Martinez-Cabrera et al. (2012), which may be consistent with our white noise results.

Others have found a lack of evidence for phylogenetic environmental niche conservatism as well. For example, Blonder et al. (2015) tested the evolutionary models underlying relationships among traits and environment in the Hawaiian silversword alliance and found strong support for white noise models, which they concluded was the result of rapid diversification of taxa into novel environments. For plant radiations in the CFR, a lack of phylogenetic niche conservatism may even be characteristic.

Diversification among environmental niches has contributed to species diversification both in *Pelargonium* (Martinez-Cabrera and Peres-Neto 2013) and *Babiana* (Schnitzler et al. 2012).

INTEGRATED TRAIT EVOLUTION

Analysis of both contemporary and evolutionary trait associations provides evidence for covariation of morphological traits and environmental variables in *Protea*. Individual morphological traits and environmental niche variables are used to indicate significant aspects of the "whole organism" and "n-dimensional hypervolume" environmental niche variation (Hutchinson 1957; Reich et al. 2003; Reich and Cornelissen 2014). Correlations among morphological traits are one way of measuring the degree to which phenotypes are integrated, whether as a result of shared function, development, or genetics (Pigliucci 2003). Similarities or differences between contemporary and evolutionary associations between morphological traits may provide clues to the causes of phenotypic integration. For example, the strong negative associations between leaf nitrogen and leaf carbon-to-nitrogen ratio and between height and canopy area in both sets of analyses could reflect fundamental biophysical constraints. In contrast, the negative association between lma and $\delta^{13}C$ (sclerophyllous leaves with higher lma have more negative $\delta^{13}C$ values (less water use efficient)) may reflect a functional association between strategies enhancing resource conservation and those enhancing water use efficiency.

Some patterns of trait integration in *Protea* are consistent with those previously seen in global datasets. In plants, several different syndromes of integrated traits have been proposed including Grime's C-S-R triangle (Grime 1988), Chapin et al.'s "stress-resistance syndrome" (Chapin III et al. 1993), Westoby's LHS strategy (1998), and the many variations of the worldwide leaf (or whole plant) economics spectrum (LES) (Wright et al. 2004; Reich and Cornelissen 2014). Each of these syndromes involve suites of correlated traits, combined with trade-offs among them. In *Protea,* for example, we find a positive evolutionary association between cnratio and lma and a negative association between nmass and lma, consistent with worldwide patterns in the LES. Similarly, we find a positive evolutionary

association between leafarea and plant height, between leafarea and cnratio, and between plant height and canopy area, and a negative association between leafarea and nmass, suggesting that there are integrated traits related to overall investment and plant size (Fig. 4A).

Not surprisingly, several environmental niche variables also covary. Since the environments descendants occupy are, at best, weakly correlated with those of their progenitors, these associations probably reflect intrinsic physical climate correlations rather than correlated niche evolution. For example, the positive network of mat, tmax, and tmin, and their negative associations with elevation are expected due to adiabatic cooling and would likely be detected in any random sample of geographic locations. In contrast, the associations between seasonality, precipitation, and temperature reflect niche hypervolumes characteristic of the CFR and perhaps of other Mediterranean climate regions around the world.

ENVIRONMENTAL FILTERING VS ENVIRONMENTAL ADAPTATION

Divergence order tests failed to provide a clear indication of whether divergence in traits preceded divergence in environmental niche or vice versa. There was weak support for divergence in morphology before divergence in environmental niche for wood density (environmental filtering, Fig. 1C) and the opposite for stomatal density (environmental adaptation, Fig. 1C). No comparisons were significant after false discovery rate correction. The weak pattern in wood density is consistent with patterns in other groups in the CFR, where differences in morphology may evolve before lineages occur in different habitats or climates, though major geological shifts in climate do not consistently facilitate radiation (Hoffmann et al. 2015). In an analysis of schoenoid sedges, for example, Slingsby and Verboom (2006) found that closely related and morphologically similar species often occur in different habitats, while more distantly related and morphologically dissimilar species often occur in similar habitats. Communities of Proteaceae, in particular, often include distantly related species with different leaf morphologies within a site (Cody 1986). More broadly, early divergences in morphological traits are

consistent with Ackerly et al.'s (2006) findings where physiological/morphological traits diverged early in the evolution of *Ceanothus* while niche/habitat differences arose throughout the phylogeny.

This result is consistent with previous work in *Protea* suggesting that trait-environment associations are adaptive. For example, Carlson et al. (2011) showed in the white proteas (a strongly supported clade of six species) that trait-performance differences in two experimental gardens are consistent with patterns expected from trait-environment associations in wild populations. That work provided direct evidence that many trait-environment associations are adaptive. Similarly, selection gradient analyses using an estimate of lifetime seed production as a proxy for fitness demonstrated differential selection in two wild populations consistent with predictions of trait-environment associations in *P. repens* (Carlson et al. 2015).

INCORPORATING UNCERTAINTY IN COMPARATIVE ANALYSES

Two sources of uncertainty should be recognized in any comparative analysis: (1) uncertainty about trait values for the species that arises because of trait variation within species and (2) uncertainty about species relationships that arises because phylogenetic relationships are imperfectly estimated. In our case, incorporating these sources of uncertainty did not qualitatively affect our results in the BayesTraits analyses or divergence order tests. The median values incorporating both intraspecific trait variation and phylogenetic uncertainty (i.e., the 100×100 analyses) were very similar to those from the 1×1 analyses based on the species mean value and the "best" phylogenetic tree. Nonetheless, the range of values generated in the 100×100 analyses indicates that results using any single point estimate, such as a mean, must be interpreted with considerable caution. This is particularly evident in the analysis of evolutionary models for environmental niche traits, where an Ornstein-Uhlenbeck model was supported in the 1×1 analysis for mat, map, elev, rfl2mm, rflcv, summer_rain, and tmax, while a white noise model was by far the predominant choice in the 100×100 analyses for all of these traits except summer rainfall.

CONCLUSIONS

The correlated evolution of traits and environment in *Protea* provides strong evidence that broadly adaptive processes played an important role in divergence of many morphological traits during this rapid radiation. In particular, we identified two main categories of trait-environment coevolution: an association between plant size and temperature, and between leaf investment and rainfall. These are supported by consistent findings between evolutionary and contemporary associations. We were unable to provide definitive evidence for the role of environmental filtering vs. adaptation in driving these associations. There is at best weak but conflicting support for different processes in the divergence of two traits that may be important in water relations: environmental filtering for wood density and environmental adaptation for stomatal density. Phenotypic traits are relatively conserved, but in this genus, the conservatism does not appear to be accounted for by environmental niche conservatism. Overall, we find substantial evidence for broadly adaptive co-evolution among traits and environment even when incorporating both uncertainty in phylogenetic relationships and to within-species variation in trait values. Future work on trait and physiological differentiation in closely related co-occurring species of *Protea* may provide more robust evidence for the mechanisms underlying these adaptive phenotype-environment associations.

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Author Contributions

N. Mitchell designed and carried out the analyses, was involved in field collection, and wrote this chapter.

J.E. Carlson planned and carried out much of the field work. K.E. Holsinger obtained funding and was crucially involved in giving feedback and revising this chapter.

Tables

Table 1. Trait and environmental variables examined in this study.

Trait	Description
height	plant height (cm)
canopy	canopy area (cm^2)
lma	leaf mass per area ($g \cdot cm^{-2}$)
wood	wood density
lwr	leaf length-to-width ratio
fwc	leaf fresh water content ($g \cdot g_{dw}^{-1}$)
sd	stomatal density (stomates·cm-^{2})
nmass	leaf nitrogen per mass (%)
d13c	leaf ^{13}C:^{12}C (‰)
cnratio	leaf carbon:nitrogen ratio

Environment	Description
mat	mean annual temperature (°C)
map	mean annual precipitation (mm)
elev	elevation (m)
pet	mean annual potential evapotranspiration (mm)
rfl2mm	days with > 2mm rain (days)
rflcv	Inter-annual coefficient of variation of precipitation (%)
summer_rain	mean monthly rainfall summed across summer months, December-February (mm)
tmax	average daily maximum temperature in January (°C)
tmin	average daily minimum temperature in July (°C)
tvar	maximum – minimum temperature (°C)

Table 2. Divergence Order Test significant results from the 1×1 and 100×100 datasets.

Trait - Envi	Divergence Age (1×1)	p-value (1×1)	Divergence Age (100×100)	p-value (100×100)	Older?
lma-tmin	-2.12E-05	0.235 (NS)	2.70E-04	0.048	trait
fwc-map	3.05E-04	0.038	2.45E-04	0.063 (NS)	trait
fwc-rfl2mm	1.92E-04	0.097 (NS)	2.59E-04	0.048	trait
fwc-tmin	1.20E-04	0.121 (NS)	3.72E-04	0.018	trait
fwc-tvar	3.27E-04	0.025	2.57E-04	0.058 (NS)	trait
wood-map	4.54E-04	0.002	4.37E-04	0.006	trait
wood-pet	3.29E-04	0.028	2.64E-04	0.036	trait
wood-rflcv	1.59E-04	0.087 (NS)	2.60E-04	0.039	trait
wood-rfl2mm	3.60E-04	0.011	3.75E-04	0.027	trait
wood-summer_rain	4.01E-04	0.018	3.22E-04	0.020	trait
wood-tmin	3.27E-04	0.009	5.72E-04	0.004	trait
wood-tvar	3.59E-04	0.016	3.60E-04	0.023	trait
leafarea-elev	-3.17E-04	0.030	-1.51E-04	0.131 (NS)	envi
leafarea-tmax	-2.66E-04	0.049	-6.14E-05	0.200 (NS)	envi
sd-elev	-3.98E-04	0.015	-2.51E-04	0.055 (NS)	envi
sd-mat	-3.09E-04	0.024	-2.00E-04	0.085 (NS)	envi
sd-rflcv	-3.06E-04	0.043	-1.67E-04	0.108 (NS)	envi
sd-tmax	-3.13E-04	0.028	-1.31E-04	0.134 (NS)	envi
d13c-elev	-3.02E-04	0.039	-1.39E-04	0.138 (NS)	envi

Figures

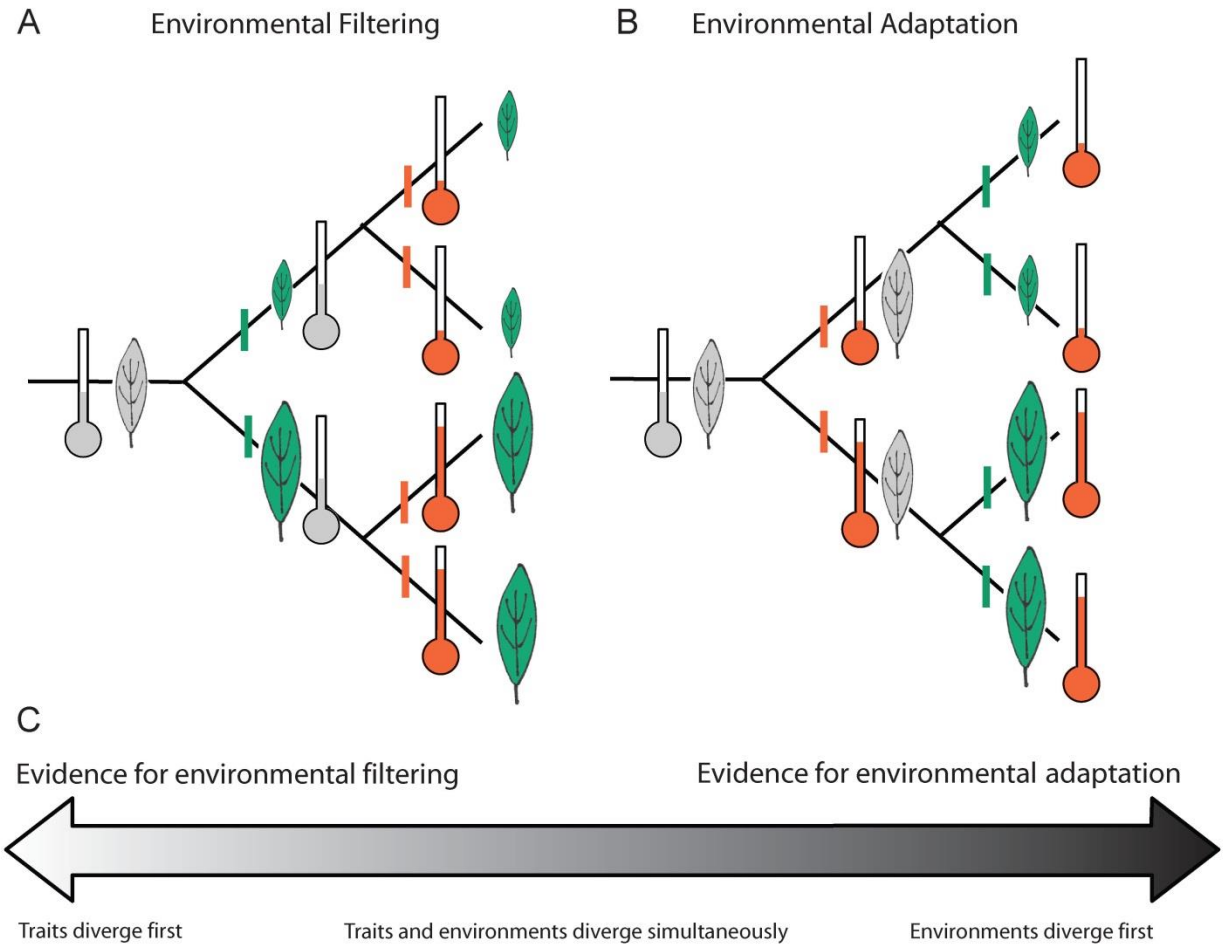


Figure 1. Diagrammatic representation of (A) environmental filtering and (B) environmental adaptation. Leaves and thermometers represent values for leaf size (trait) and temperature (environment), respectively. Gray figures are ancestral states, while green (trait) or orange (environment) figures refer to derived states and tick marks indicate changes along the tree. In (A), from some ancestral form, changes in traits occur first (while in the same environment), followed by filtering of species or lineages into new environments. In (B), changes in environments occur first (with the same trait values), followed by *in situ* adaptation of traits to the given environment. Both can result in the same contemporary trait-environment association (i.e. big leaves in cold environments, small leaves in hot environments). (C) Conceptual framework outlining expected evidence for either environmental adaptation or environmental filtering with respect to timing and match between contemporary and evolutionary associations, assuming the detection of significant trait-environment associations indicative of broad scale adaptive processes.

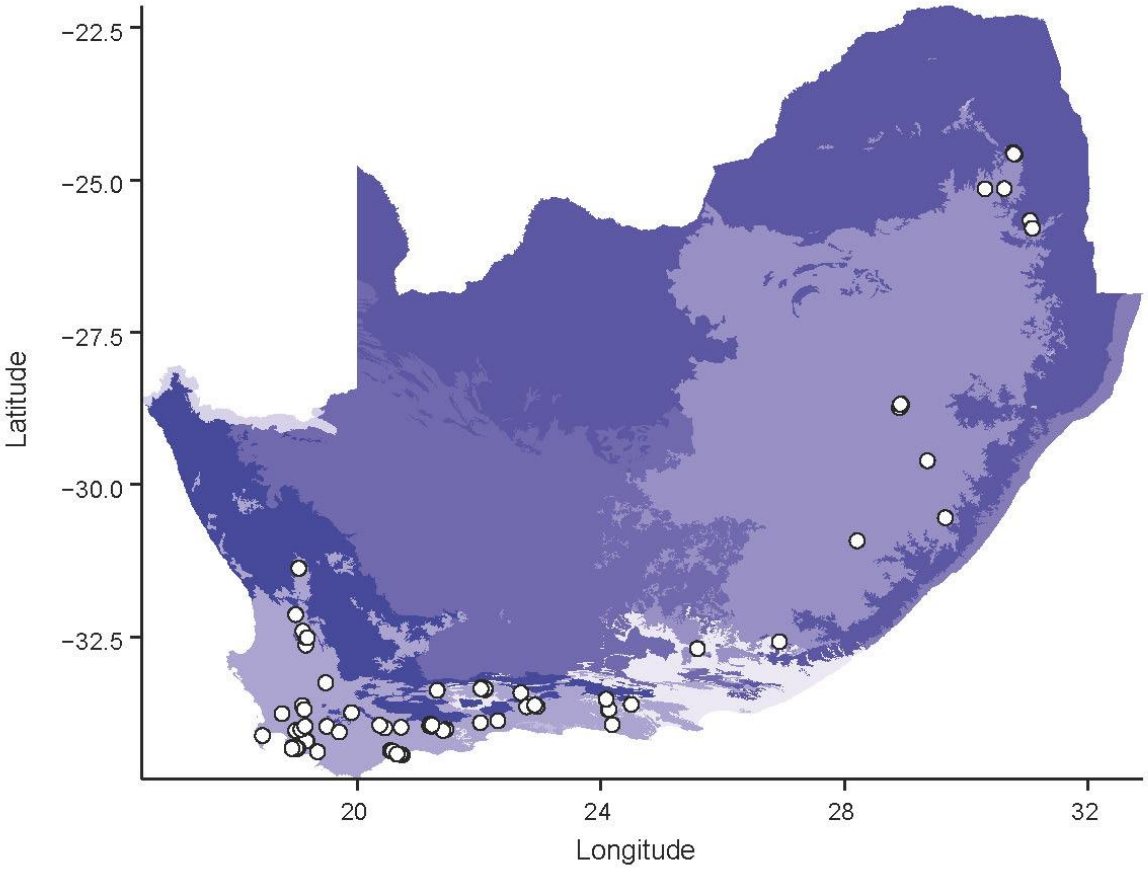


Figure 2. Map of individuals sampled for trait data for this study. Colors correspond to biomes as defined by Mucina and Rutherford (2006). Voucher and latitude/longitude data can be found in Appendix S1.

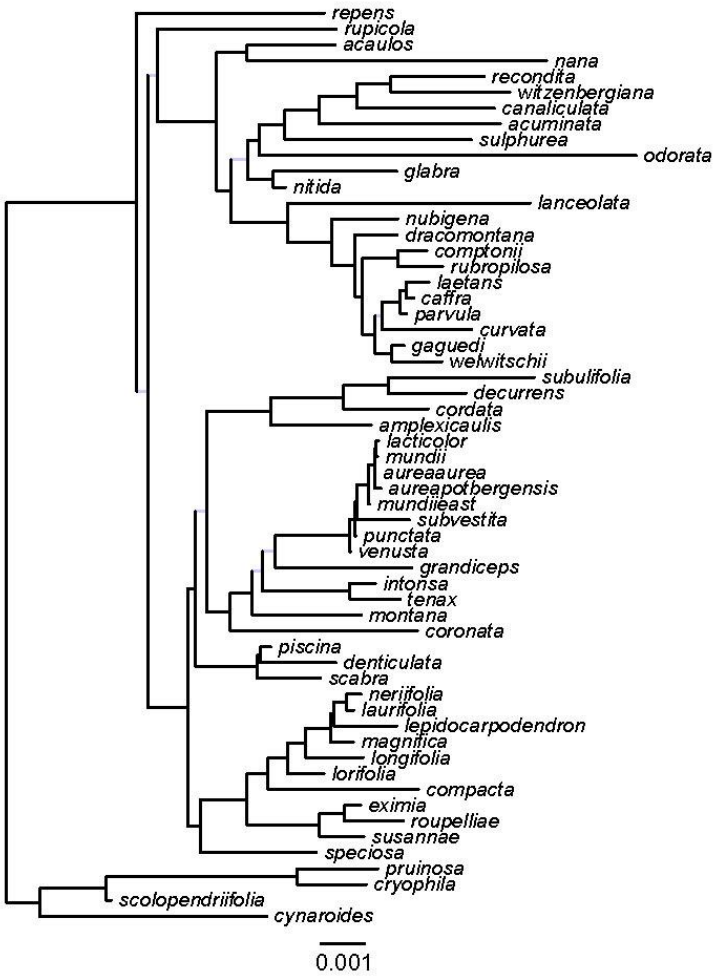


Figure 3. Phylogeny used as the "best" tree generated from ASTRAL-II in Mitchell et al. (2017). Branches black in color supported by 90% bootstrap support or higher, branches in gray have less than 90% bootstrap support. Scale bar corresponds to the number of substitutions per site.

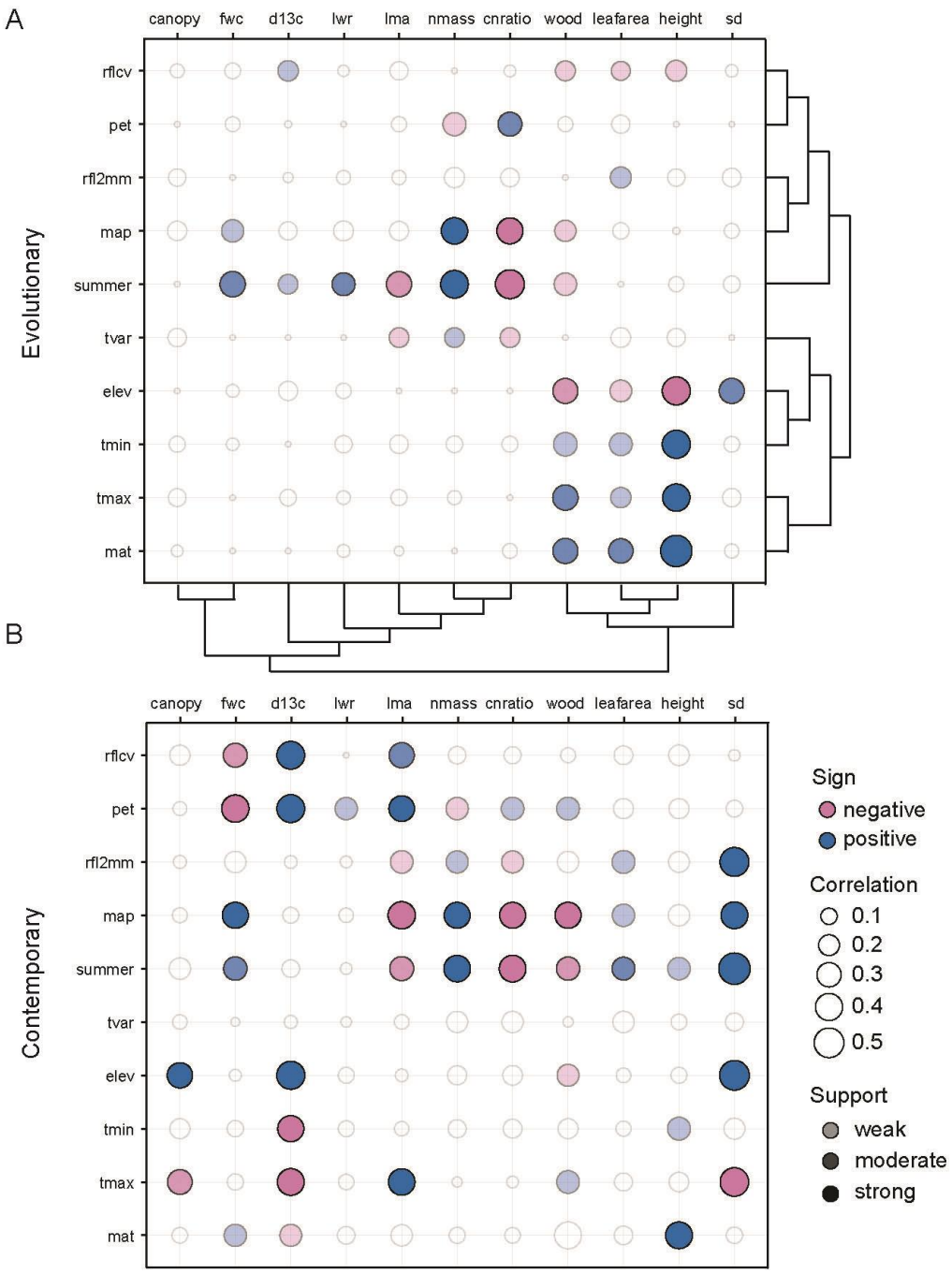


Figure 4. Trait-environment associations for (A) evolutionary (BayesTraits) (B) contemporary analyses. Correlations are either positive (blue) or negative (magenta), vary in strength (size of circle), and have different levels of support indicated by transparency of circle color (weak support: logBF > 2, $p < 0.10$, most transpaent; moderate support: logBF > 5, $p < 0.05$, medium transpaency; strong support: logBF > 10, $p < 0.01$, darkest circles). Correlations not supported at any level have no fill (completely transparent). Dendrograms for the evolutionary analyses are based on distance-matrices, and order is preserved in the contemporary data to more easily make visual comparisons.

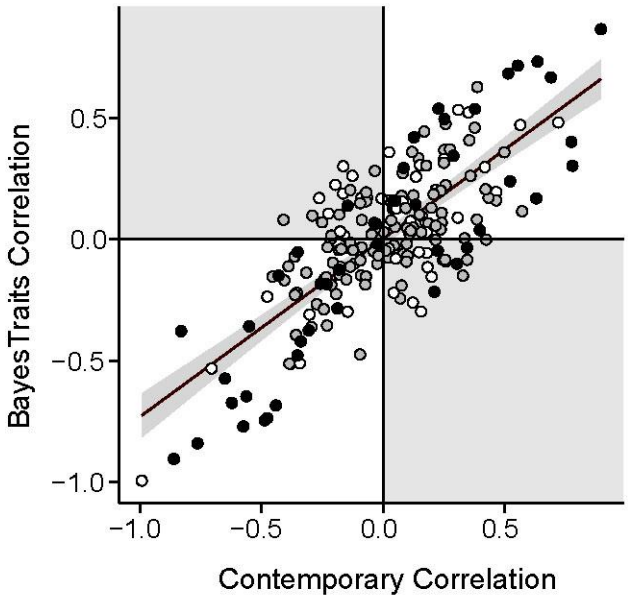


Figure 5. Contemporary vs. evolutionary correlations. Strong positive association between contemporary and evolutionary BayesTraits correlation values. Points in white quadrants are those where the sign of the relationships are the same, points in the gray quadrants are those where the sign of the relationships are different. White fill = trait-trait, gray fill = trait-envi, black fill = envi-envi comparisons.

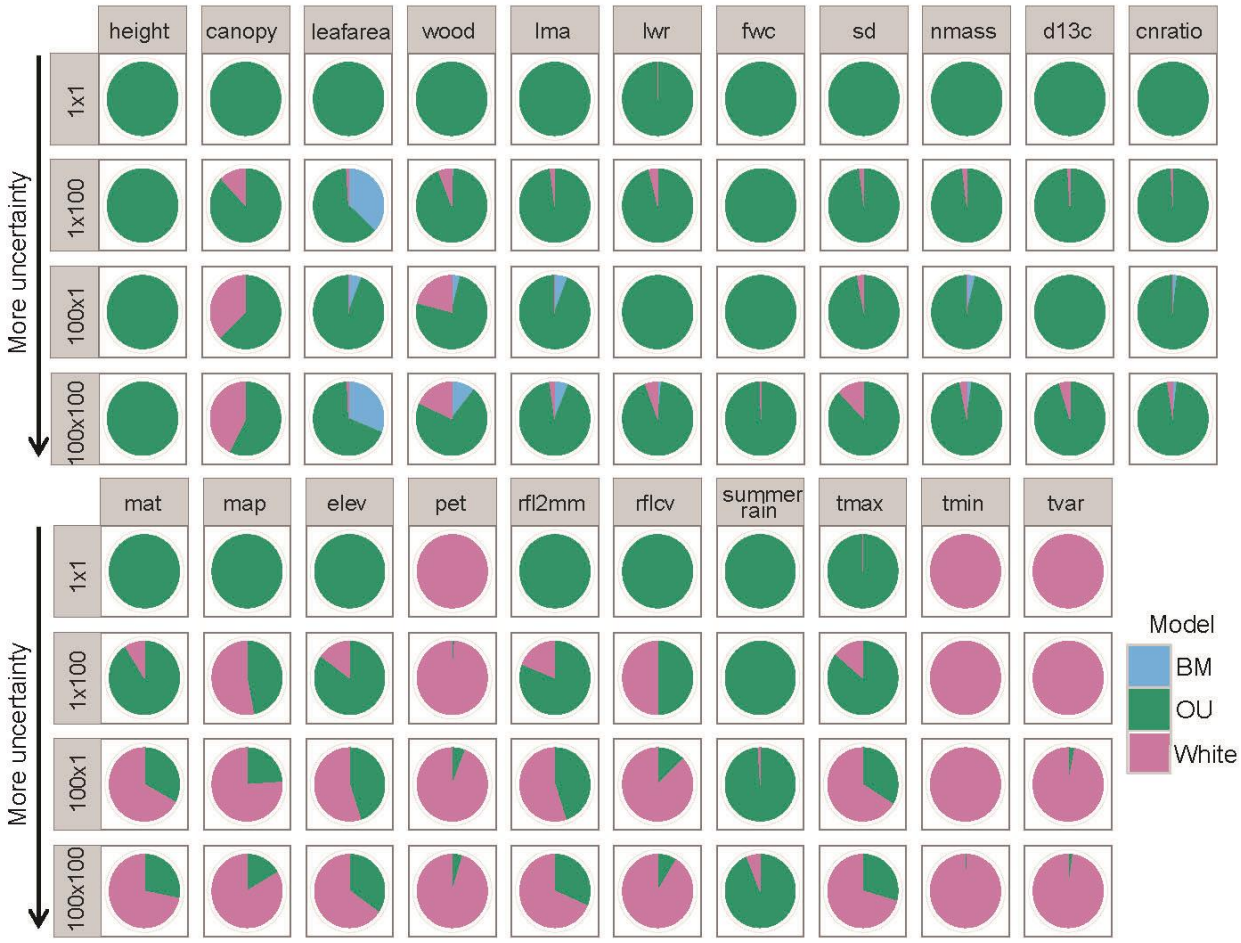


Figure 6. Models of evolution for (A) traits and (B) environmental variables. Color of the pie chart indicates the % of replicates where BM (blue), OU (green), or white noise (magenta) models of evolution have the lowest AIC value. 1×1: mean observation and best ASTRAL-II phylogeny; 1×100: mean observations × 100 bootstrap trees (n = 100); 100×1: 100 trait/envi samples x best tree (n = 100); 100×100: 100 trait or envi samples × 100 bootstrap trees (n = 10,000).

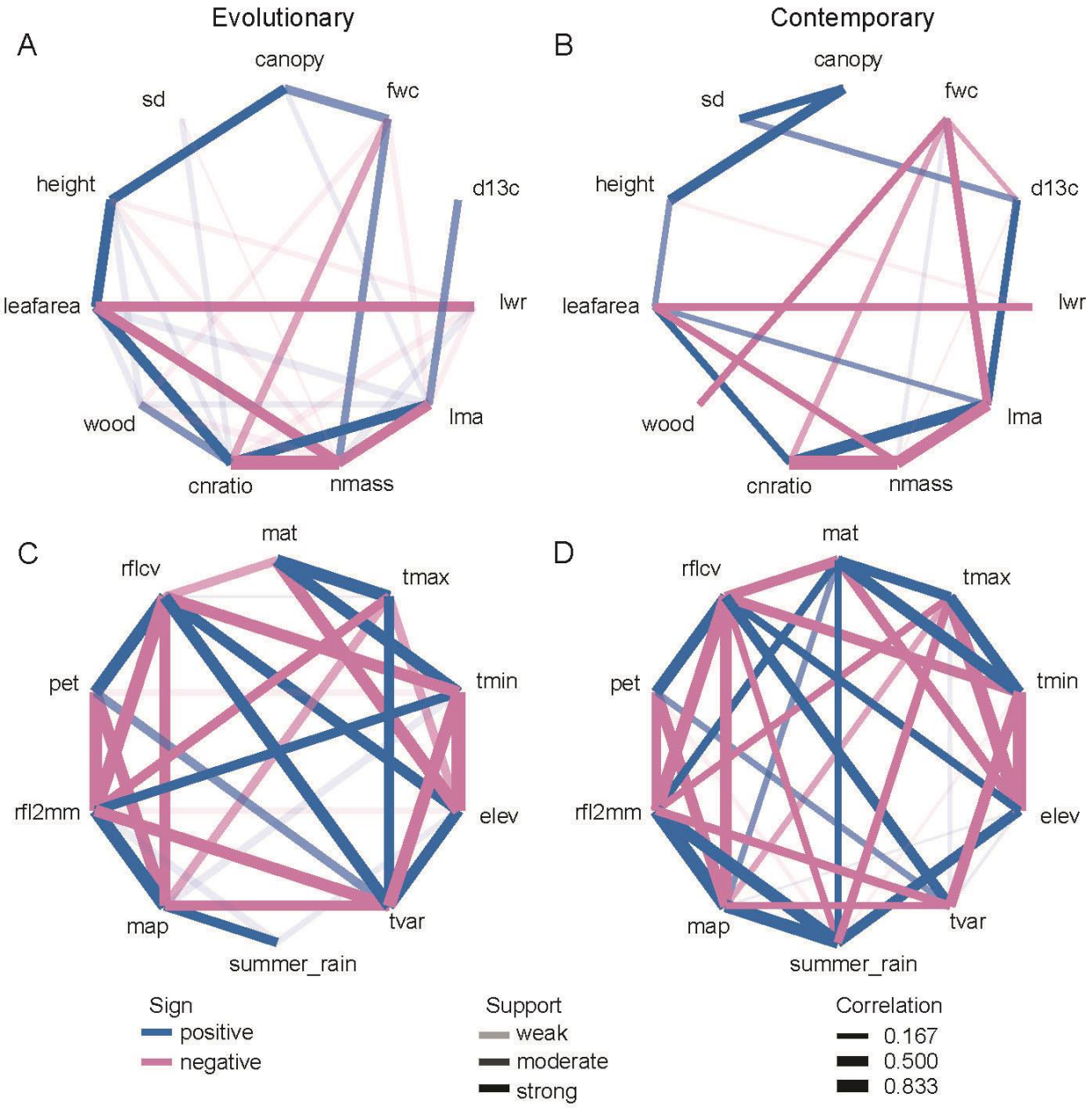


Figure 7. Trait-trait and environment-environment integration. “Schlichtograms” show the sign, strength, and significance of correlations among traits (A, B) and among environmental variables (C, D) for both evolutionary correlations based on BayesTraits (A, C) and contemporary correlations (B, D). Correlations are either positive (blue) or negative (magenta), vary in strength (width of line), and have different levels of support indicated by transparency of line (weak support: logBF > 2, $p < 0.10$, most transparent lines; moderate support: logBF > 5, $p < 0.05$, medium transparency lines; strong support: logBF > 10, $p < 0.01$, darkest lines). Correlations not supported at any level are not shown.

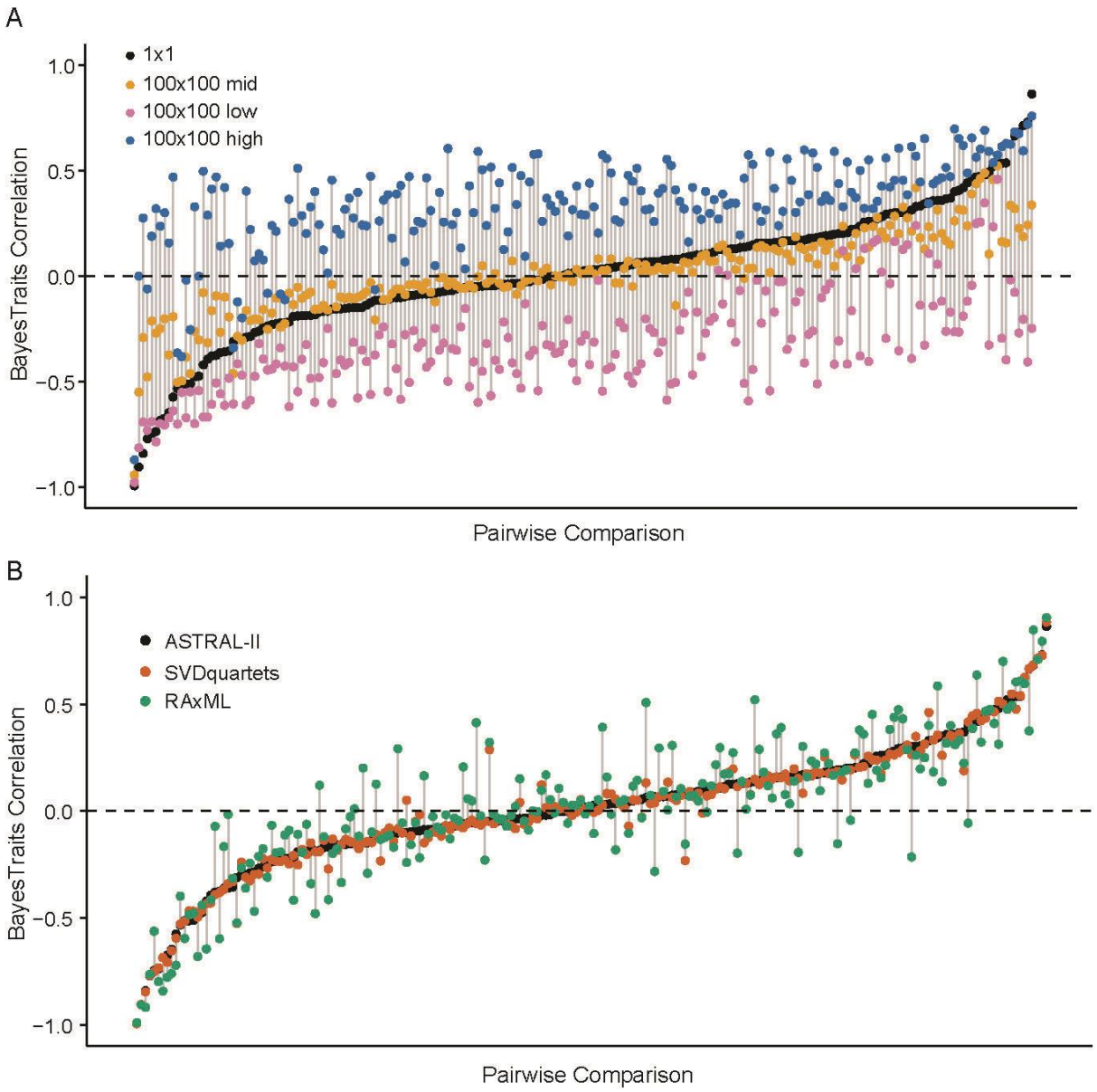
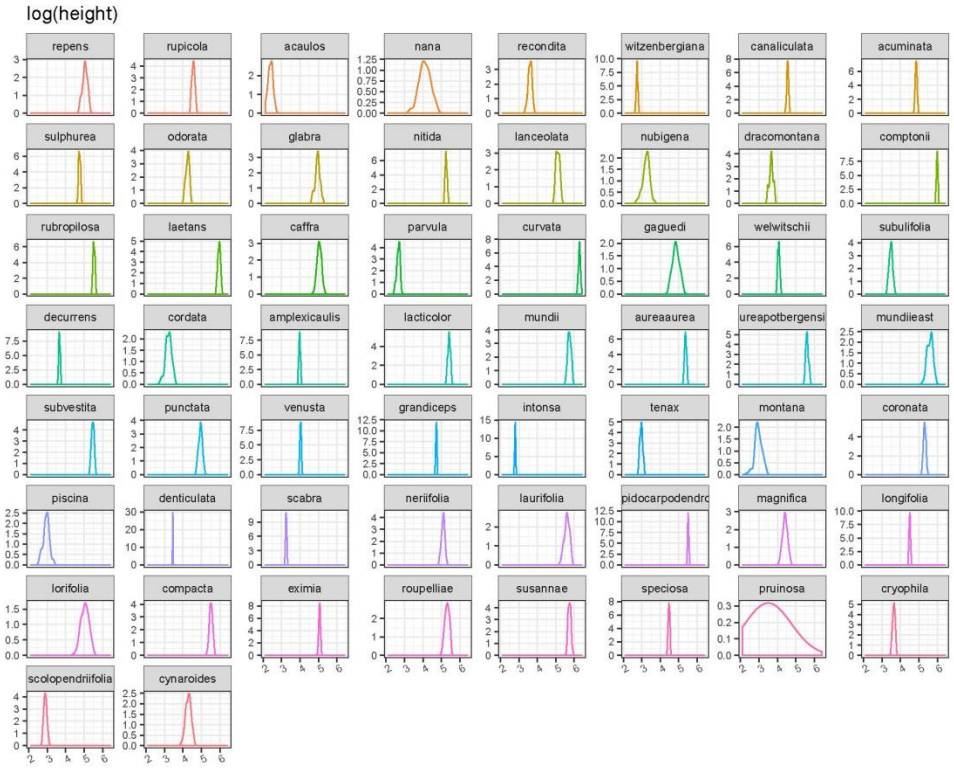


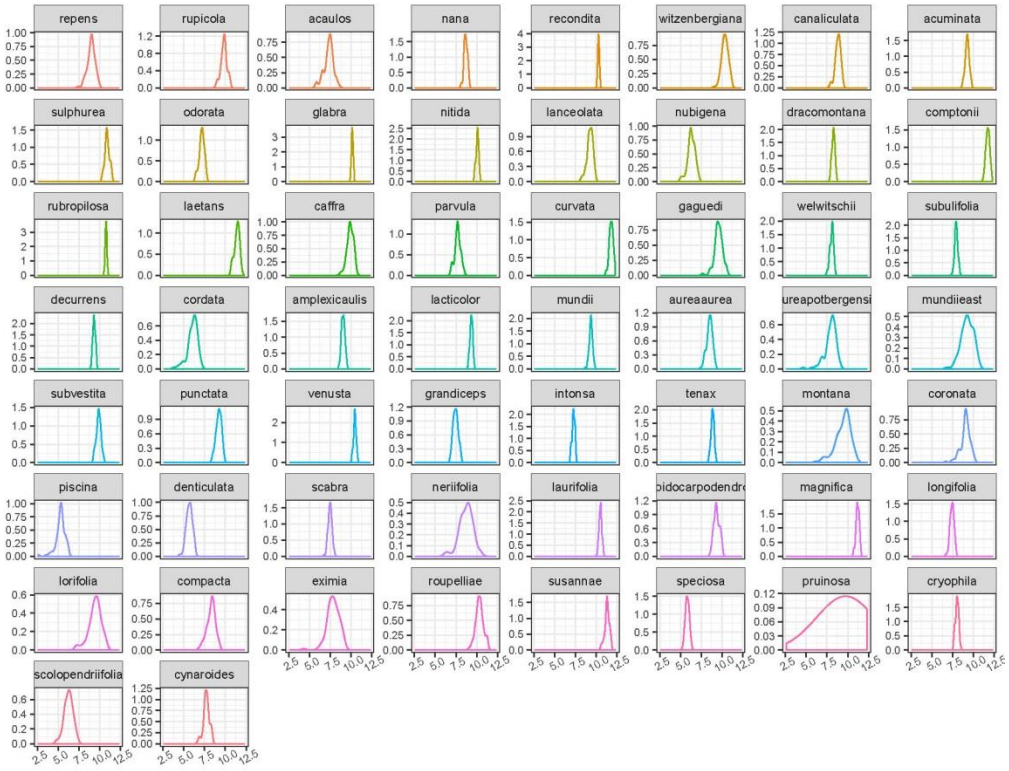
Figure 8. Uncertainty in BayesTraits analyses. (A) Comparisons of the correlation coefficients from the 1×1 (mean observation x best tree, black) with the median (gold), low (2.5%, magenta), and high (97.5%, blue) values from the 100×100 analyses for all 210 comparisons. Pairwise comparisons are arbitrarily sorted by the 1×1 correlation value. (B) Comparisons of 1×1 values from the ASTRAL-II (black), SVDquartets (orange), and RAxML (green) best species trees.

Supplemental Figures and Tables

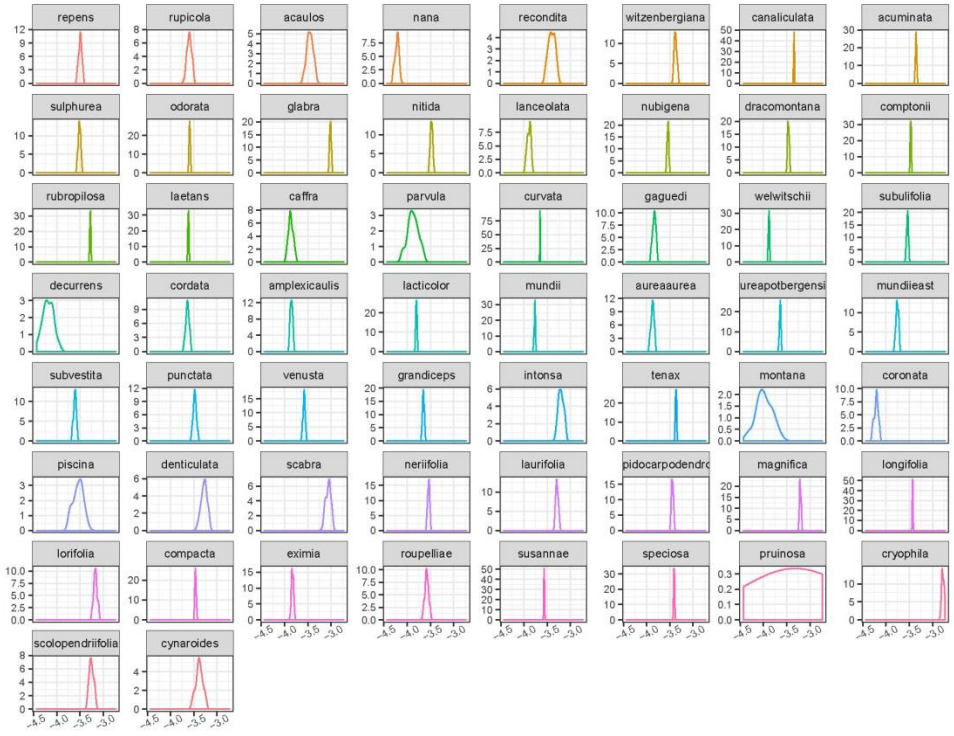
Figure S1. Density plots for posterior samples for traits and histograms for environmental variables.

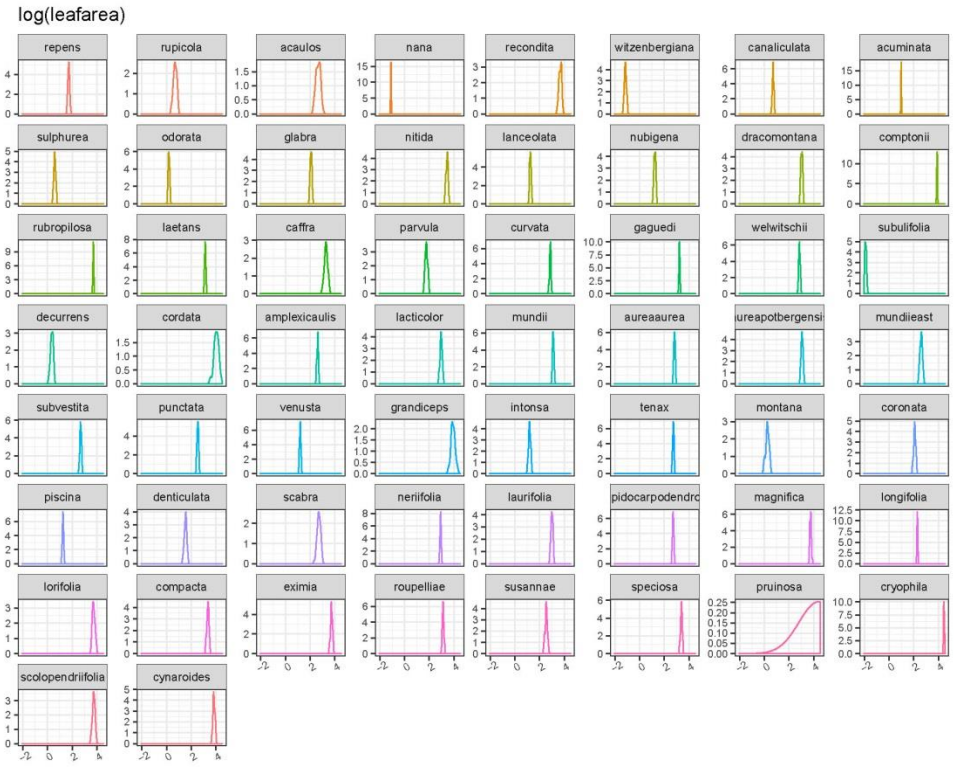


log(canopy)

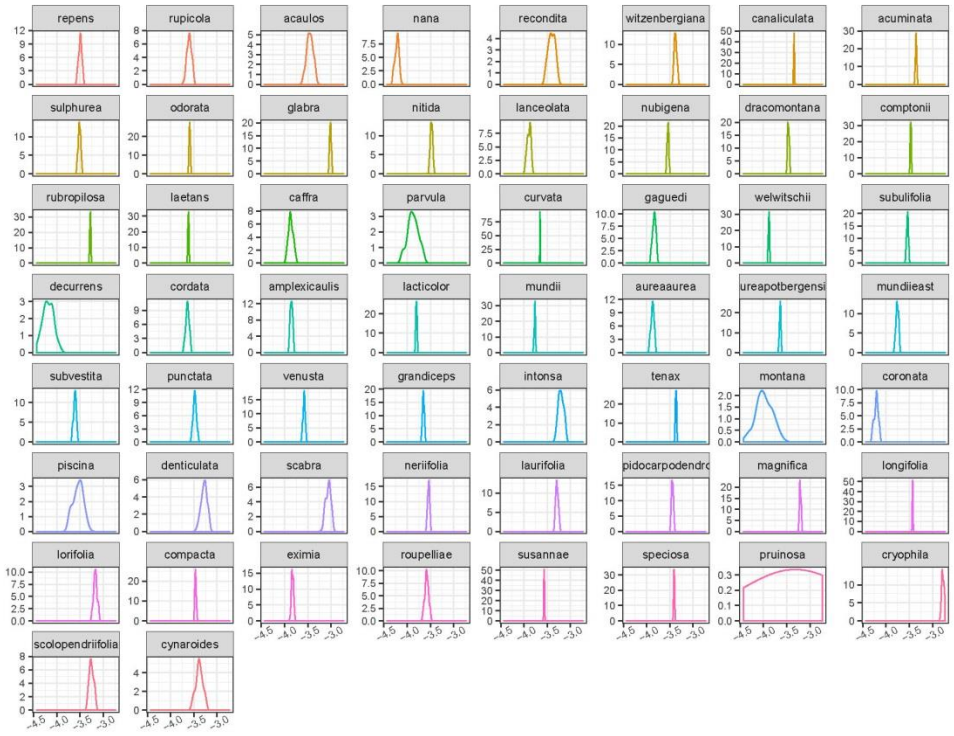


log(lma)

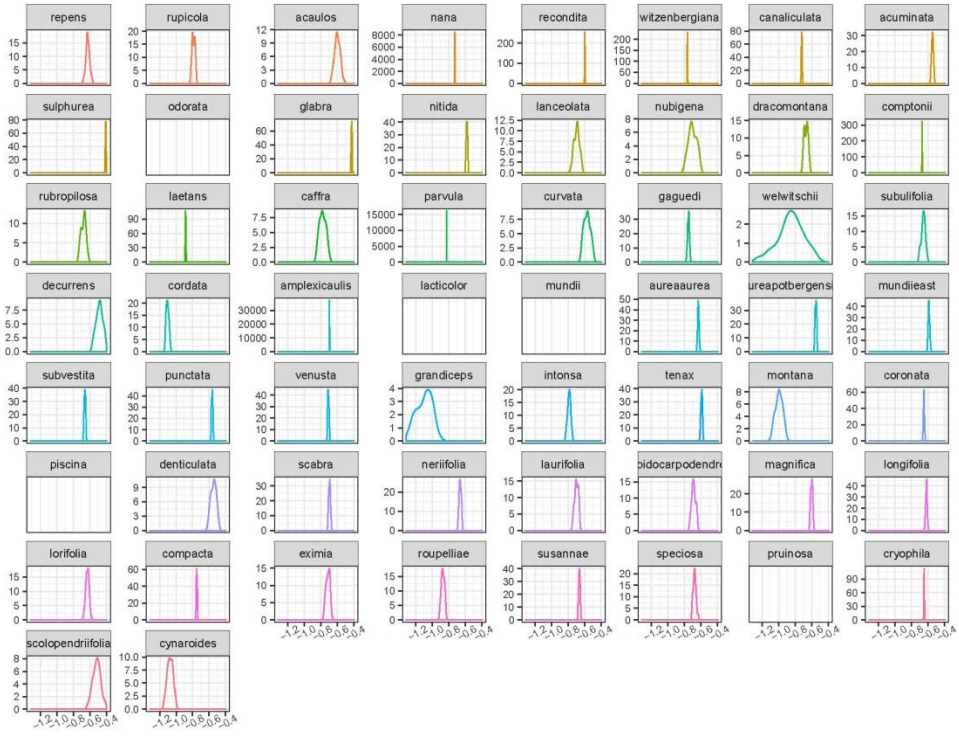



log(leafarea)
repens
rupicola
acaulos
nana
recondita
witzenbergiana
canaliculata
acuminata
sulphurea
odorata
glabra
nitida
lanceolata
nubigena
dracomontana
comptonii
rubropilosa
laetans
caffra
parvula
curvata
gaguedi
welwitschii
subulifolia
decurrens
cordata
amplexicaulis
lacticolor
mundii
aureaaurea
ureapotbergensi
mundiieast
subvestita
punctata
venusta
grandiceps
intonsa
tenax
montana
coronata
piscina
denticulata
scabra
neriifolia
laurifolia
pidocarpodendro
magnifica
longifolia
lorifolia
compacta
eximia
roupelliae
susannae
speciosa
pruinosa
cryophila
scolopendriifolia
cynaroides

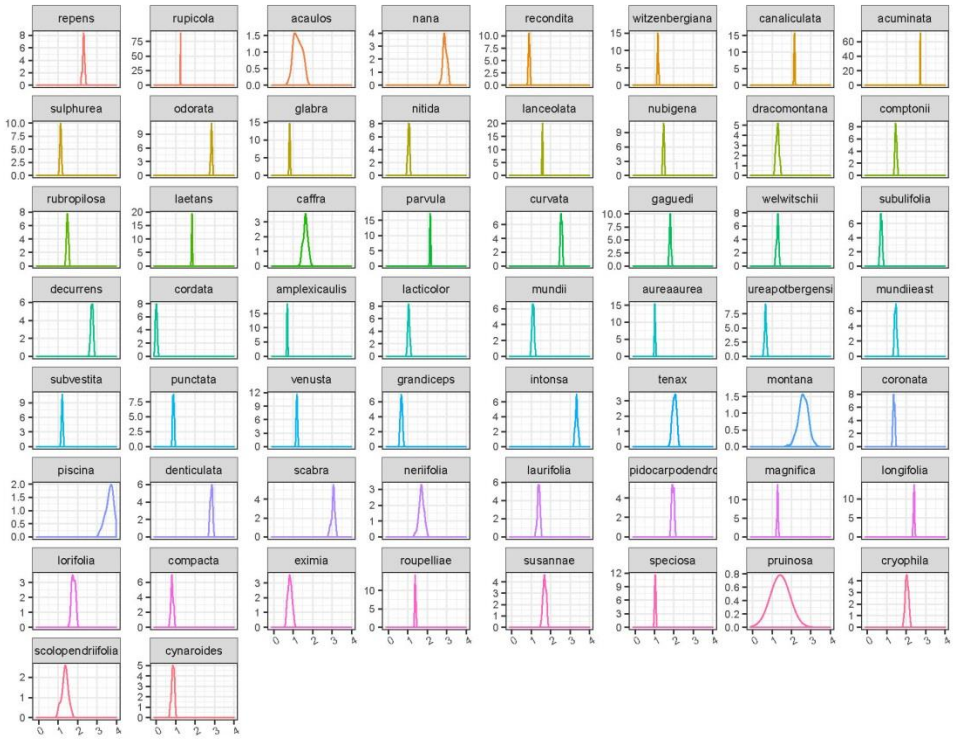
log(lma)



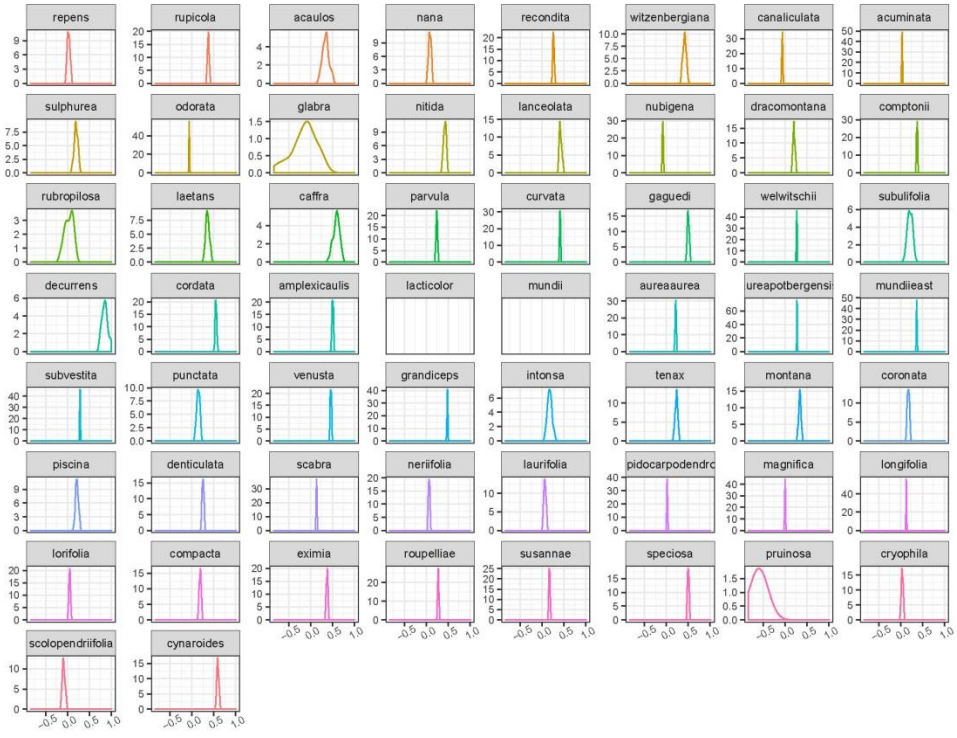
log(wood)



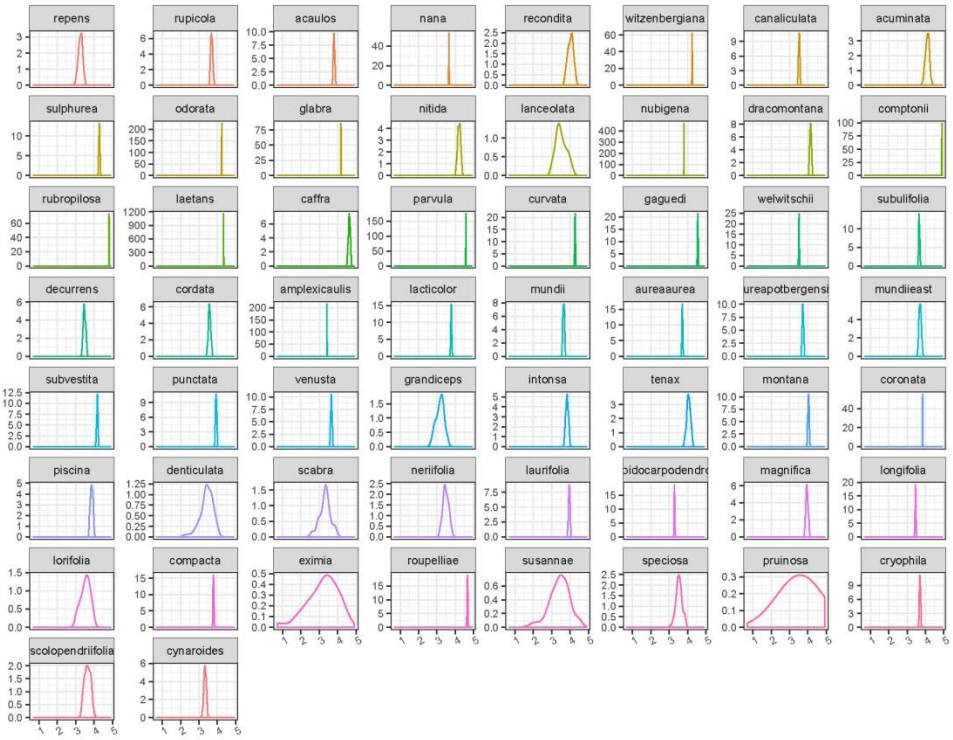
log(lwr)



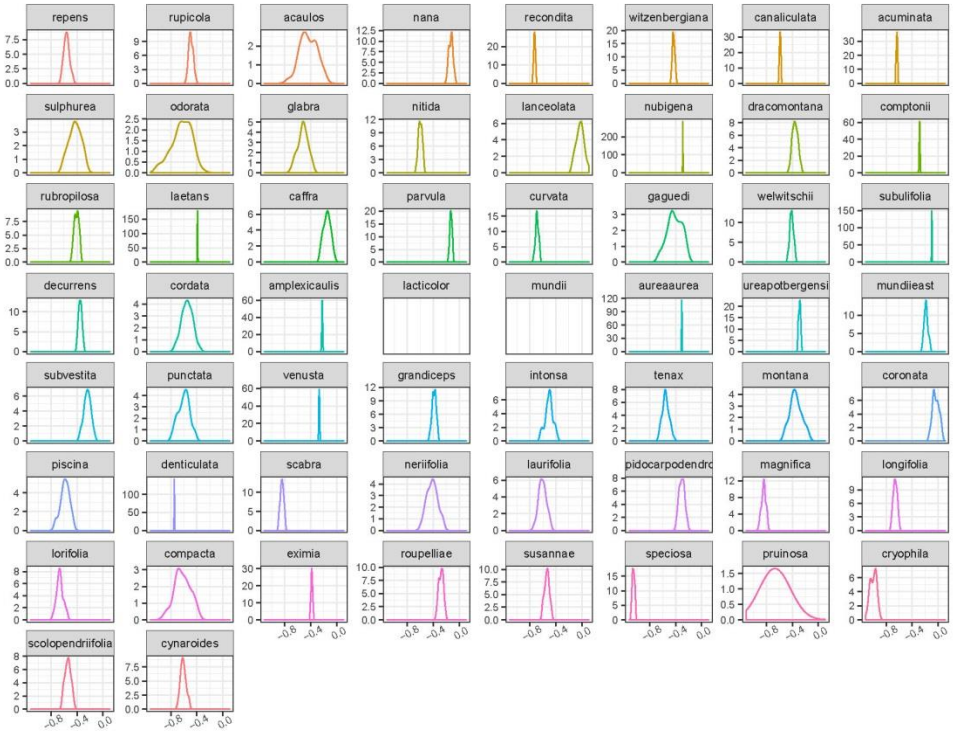
log(fwc)



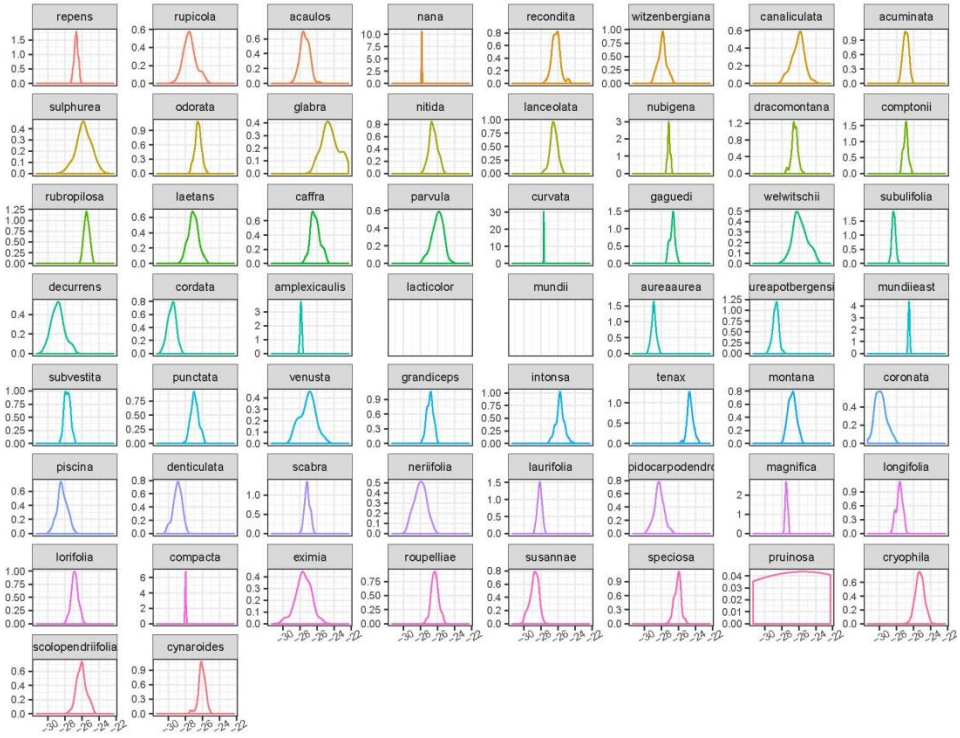
log(sd)



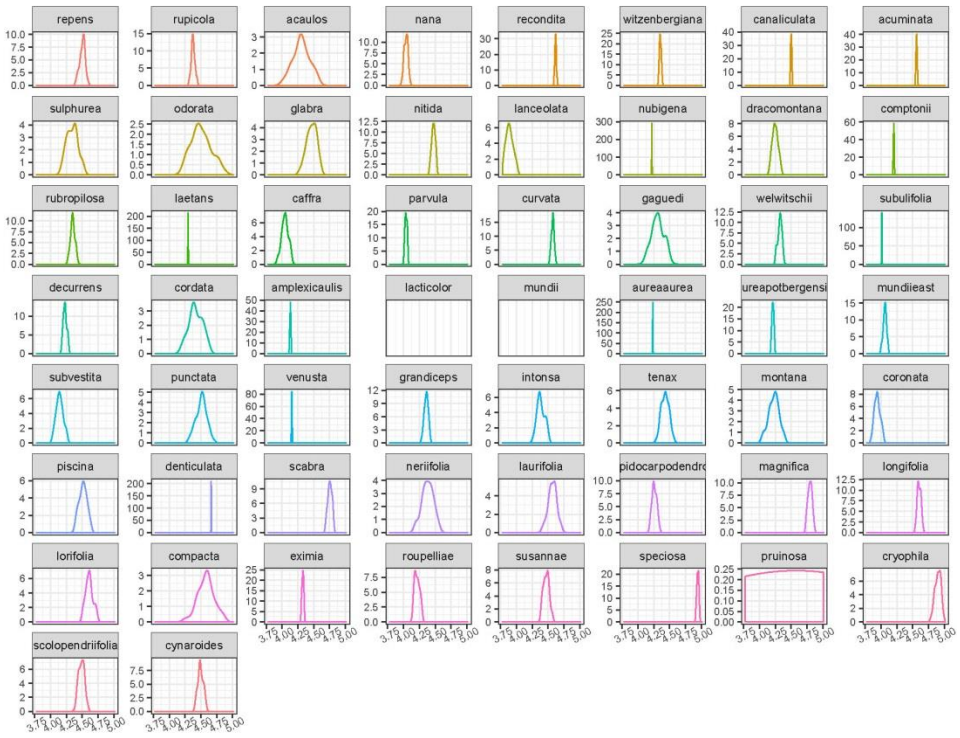
log(nmass)



d13



log(cnratio)



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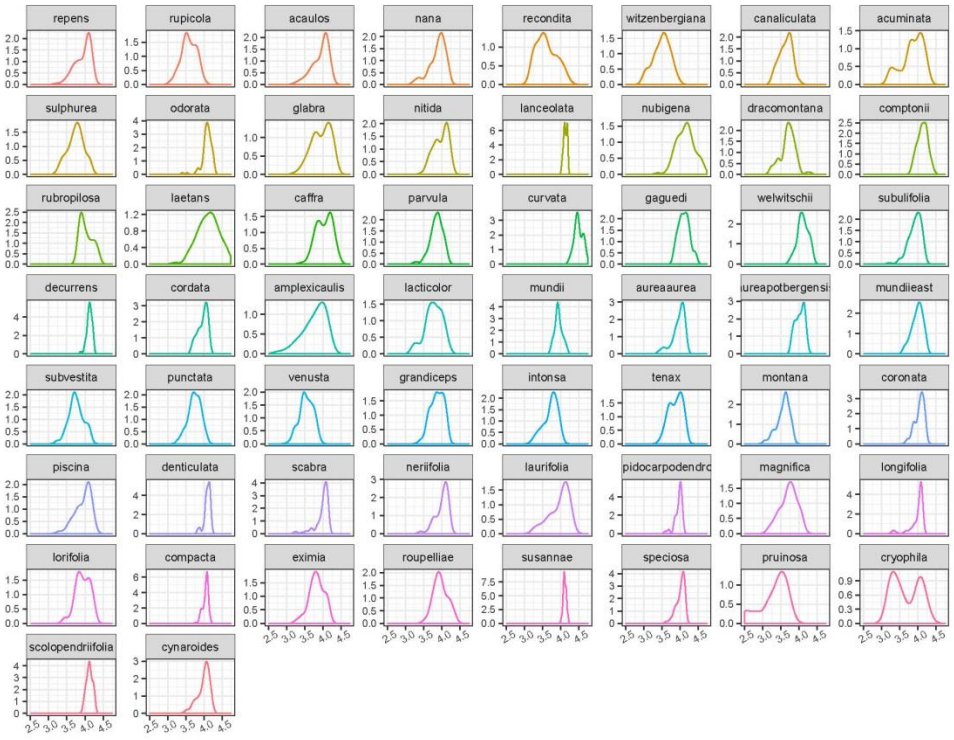
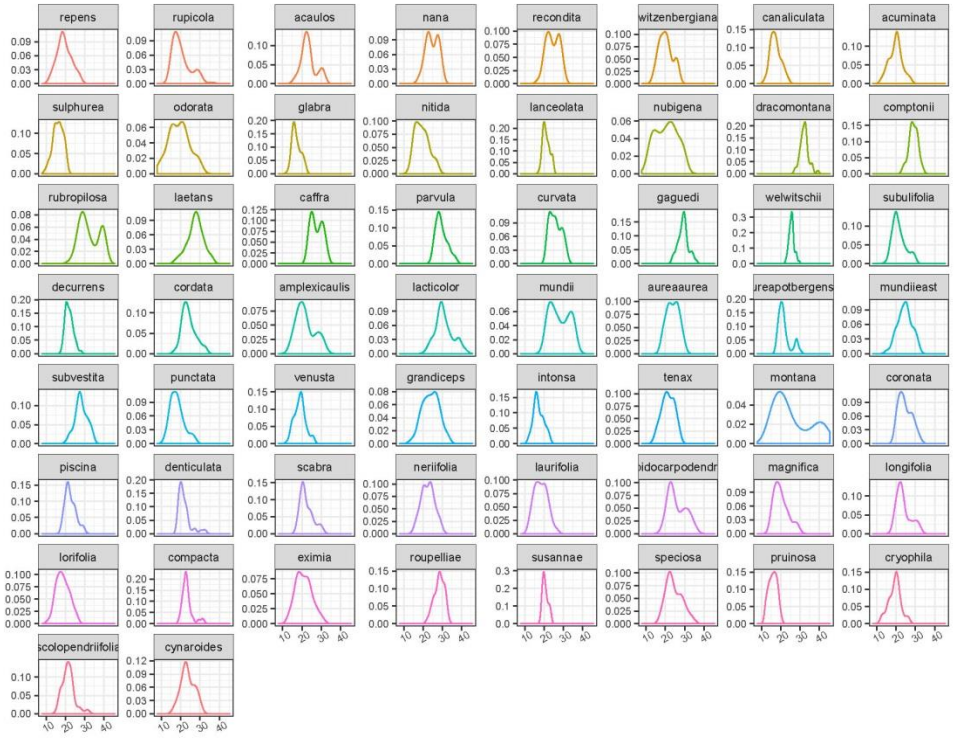
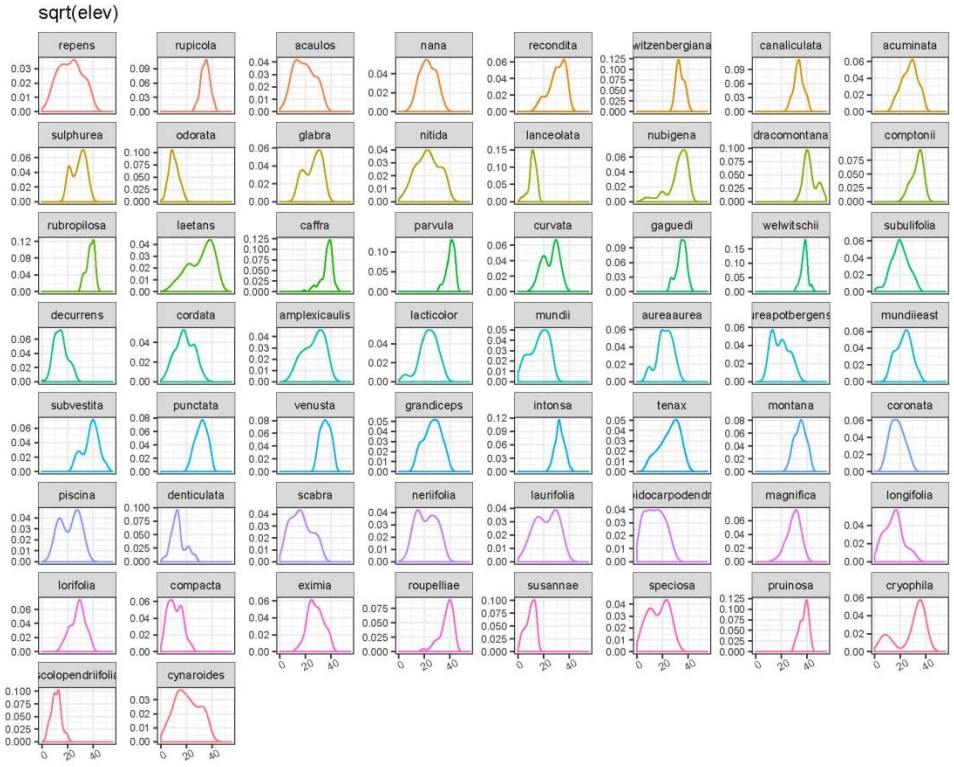
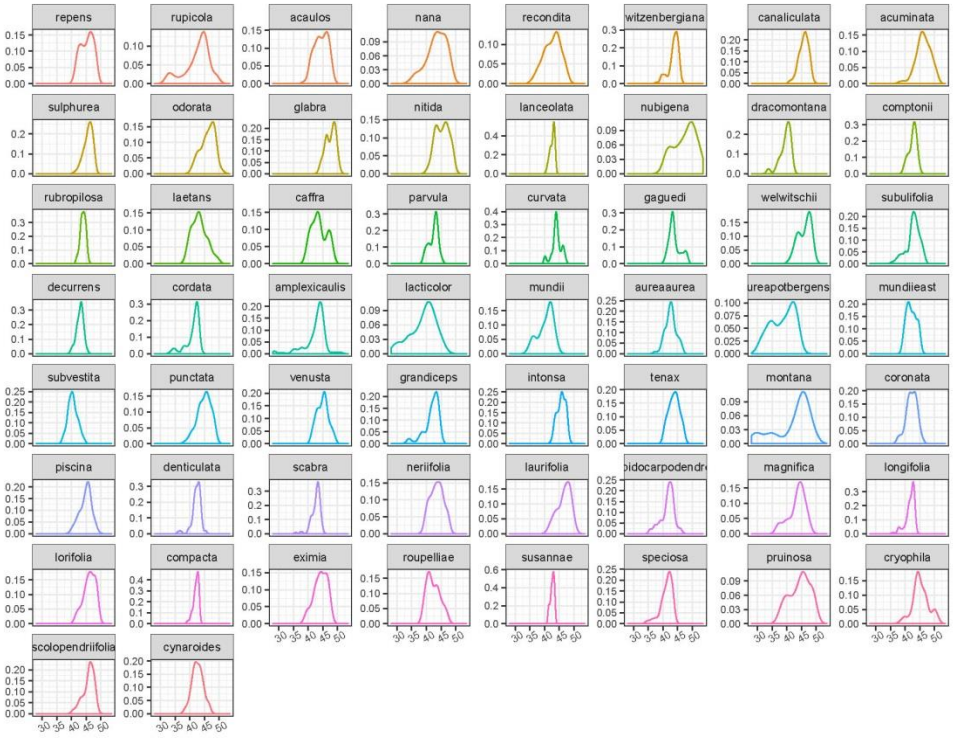


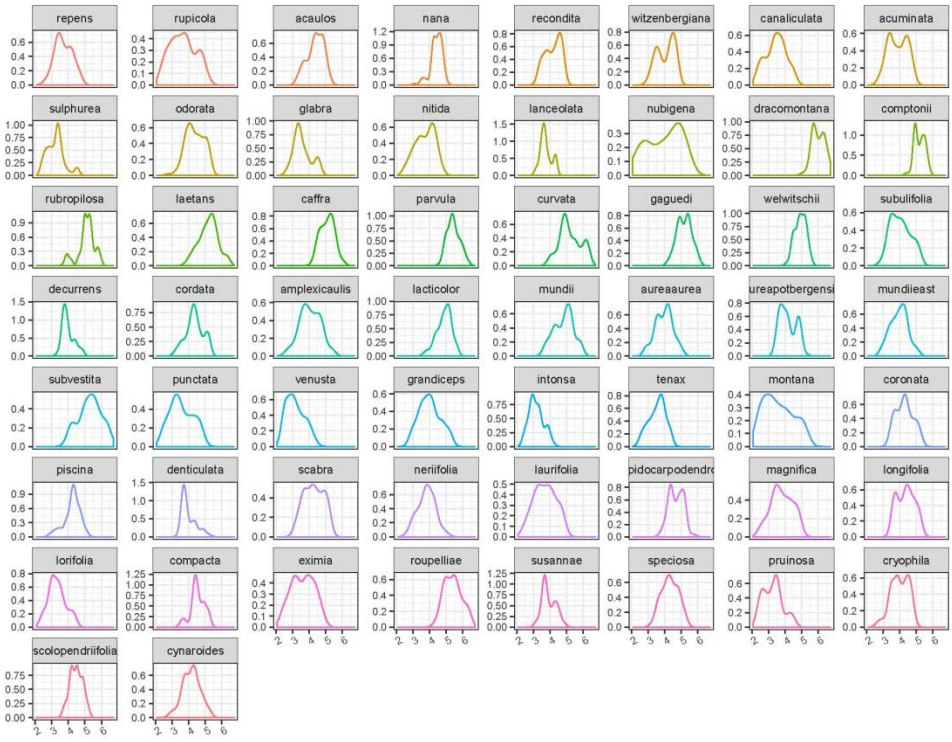
sqrt(map)



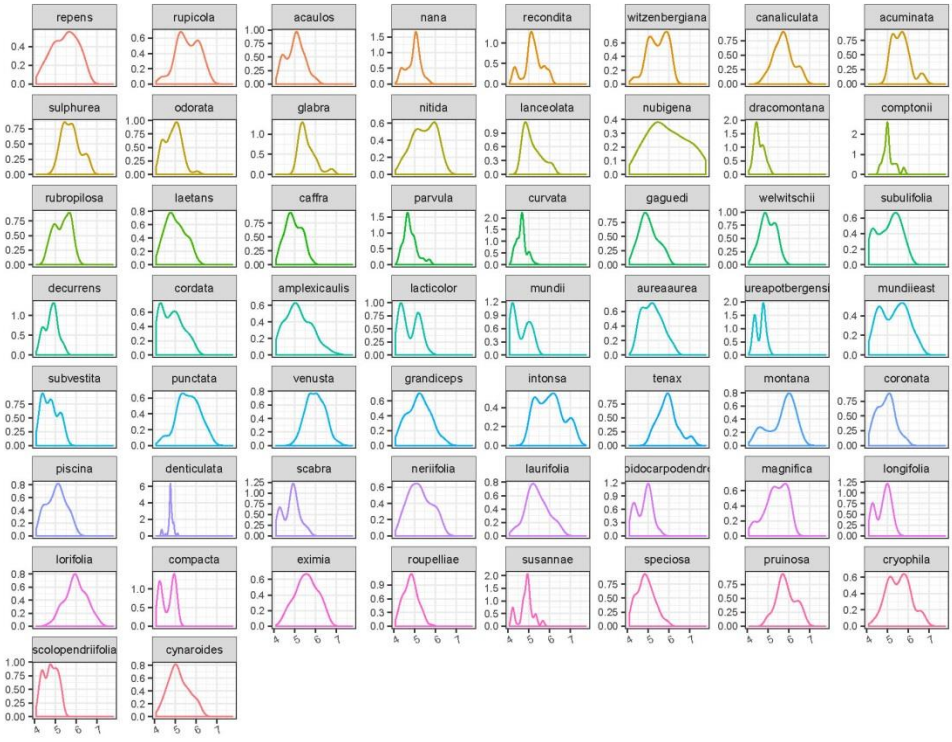

sqrt(elev)
repens
rupicola
acaulos
nana
recondita
witzenbergiana
canaliculata
acuminata
sulphurea
odorata
glabra
nitida
lanceolata
nubigena
dracomontana
comptonii
rubropilosa
laetans
caffra
parvula
curvata
gaguedi
welwitschii
subulifolia
decurrens
cordata
amplexicaulis
lacticolor
mundii
aureaaurea
ureapotbergens
mundiieast
subvestita
punctata
venusta
grandiceps
intonsa
tenax
montana
coronata
piscina
denticulata
scabra
neriifolia
laurifolia
magnifica
longifolia
lorifolia
compacta
eximia
roupelliae
susannae
speciosa
pruinosa
cryophila
cynaroides


sqrt(pet)
repens
rupicola
acaulos
nana
recondita
witzenbergiana
canaliculata
acuminata
sulphurea
odorata
glabra
nitida
lanceolata
nubigena
dracomontana
comptonii
rubropilosa
laetans
caffra
parvula
curvata
gaguedi
welwitschii
subulifolia
decurrens
cordata
amplexicaulis
lacticolor
mundii
aureaaurea
ureapotbergens
mundiieast
subvestita
punctata
venusta
grandiceps
intonsa
tenax
montana
coronata
piscina
denticulata
scabra
neriifolia
laurifolia
magnifica
longifolia
lorifolia
compacta
eximia
roupelliae
susannae
speciosa
pruinosa
cryophila
cynaroides

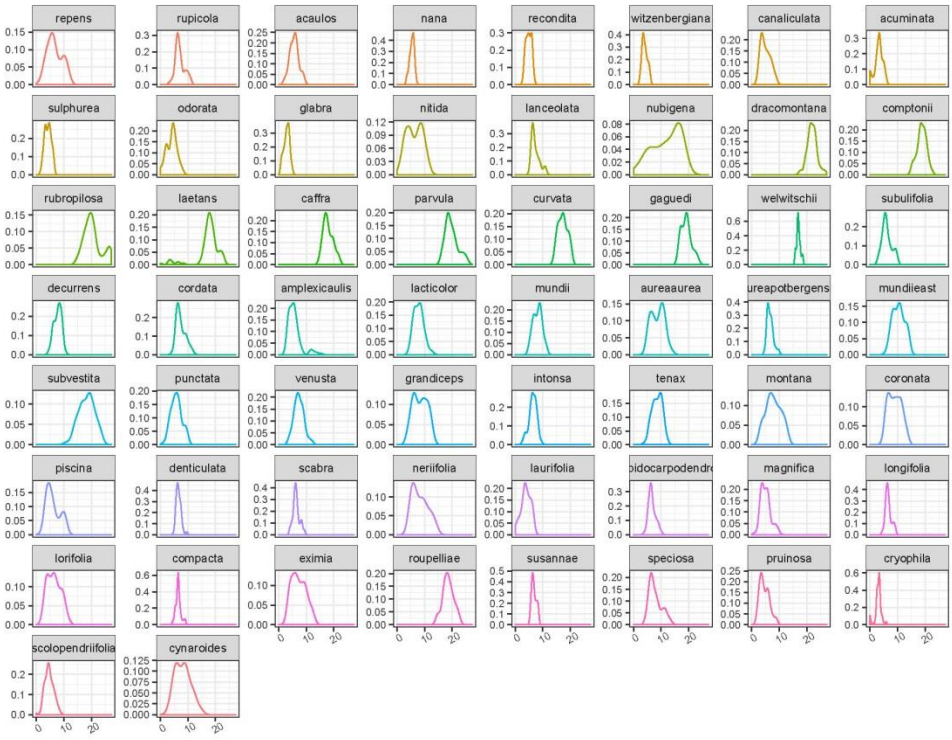
sqrt(rfl2mm)



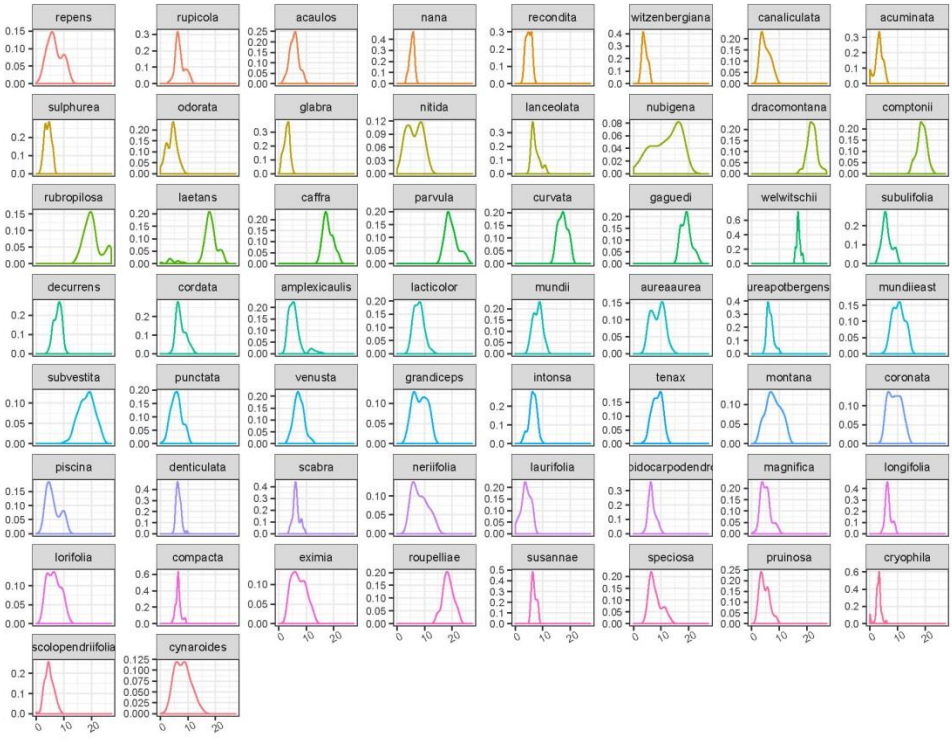
sqrt(rflcv)



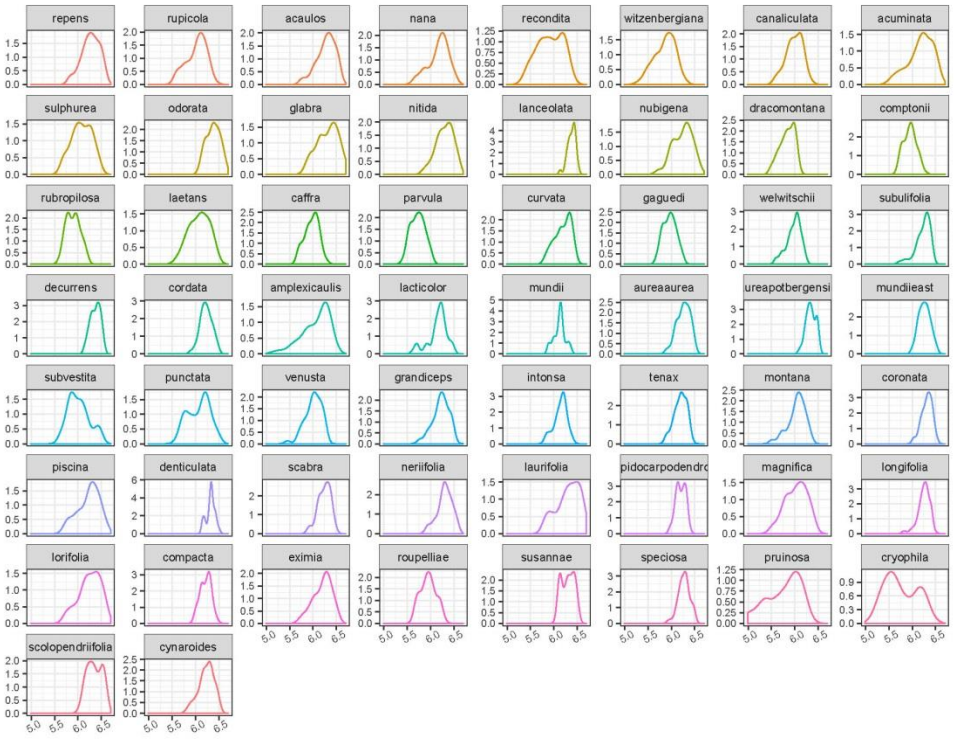
sqrt(summer)



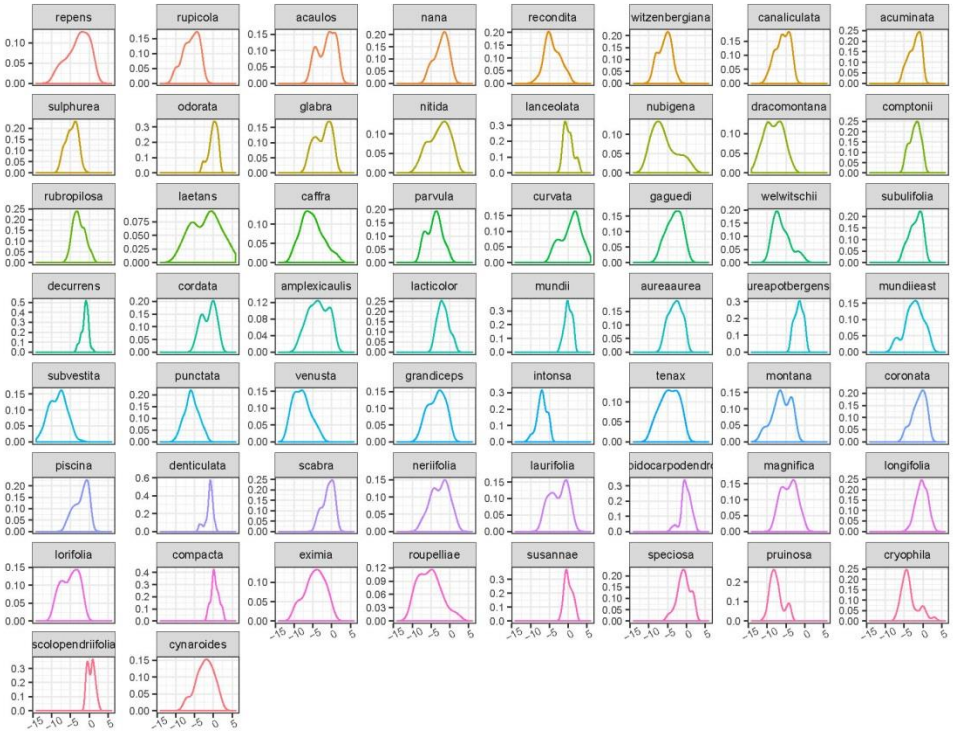
sqrt(summer)



sqrt(tmax)



(tmin)



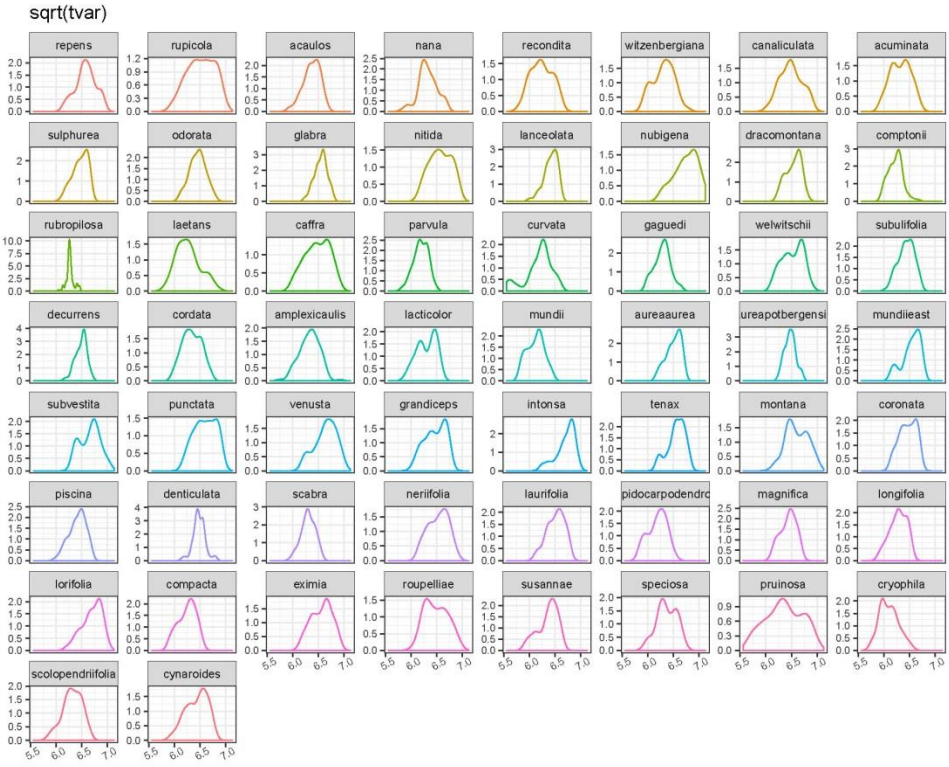

sqrt(tvar)
repens
rupicola
acaulos
nana
recondita
witzenbergiana
canaliculata
acuminata
sulphurea
odorata
glabra
nitida
lanceolata
nubigena
dracomontana
comptonii
rubropilosa
laetans
caffra
parvula
curvata
gaguedi
welwitschii
subulifolia
decurrens
cordata
amplexicaulis
lacticolor
mundii
aureaaurea
ureapotbergensi
mundiieast
subvestita
punctata
venusta
grandiceps
intonsa
tenax
montana
coronata
piscina
denticulata
scabra
neriifolia
laurifolia
pidocarpodendrc
magnifica
longifolia
lorifolia
compacta
eximia
roupelliae
susannae
speciosa
pruinosa
cryophila
scolopendriifolia
cynaroides

Table S1. Sampling information for DNA used in this study. Vouchers stored at the University of Connecticut George Safford Torrey Herbarium (CONN).

Species_name	Authority	Conn Accession #	Site_location	Site_number	Latitude	Longitude
acaulos	(L.) Reichard	227594	Kogelberg_1	PR_2026	-34.3199	18.96021
acaulos	(L.) Reichard	227586	Uitkyk_Pass_2	PR_2042	-32.4068	19.10799
acuminata	Sims	227590	Sneeuberg_Hut_1	PR_2035	-32.4891	19.15159
amplexicaulis	(Salisb.) R. Br.	256139	Bains_Kloof	PR_2059	-33.6286	19.10092
aureaaurea	(Burm. f.) Rourke	227568	Garcia_Pass_1	PR_2018	-33.9572	21.21965
aureaaurea	(Burm. f.) Rourke	141498	DR	DR	-33.8778	22.31026
aureaaurea	(Burm. f.) Rourke	141487	LW	LW	-33.7435	19.90748
aureaaurea	(Burm. f.) Rourke	141483	MP	MP	-33.9963	20.45651
aureaaurea	(Burm. f.) Rourke		PV	PV	-33.9502	20.37055
aureaaurea	(Burm. f.) Rourke	141459	RP	RP	-33.9087	22.02348
aureaaurea	(Burm. f.) Rourke	141488	RV	RV	-34.0628	19.70707
aureapotbergensis	Rourke	230305	Potberg_Trail_2	PR_2004	-34.3763	20.57062
aureapotbergensis	Rourke	141482	KP	KP	-34.3791	20.58325
aureapotbergensis	Rourke	141481	PB	PB	-34.4226	20.65524
caffra	Meisn.	248053	Blyde_River_1	PR_2043	-24.576	30.79812
caffra	Meisn.	245131	Songmivelo_2	PR_2051	-25.795	31.08337
caffra	Meisn.	248100	Royal_Natal_5	PR_2056	-28.6845	28.93657
canaliculata	Andrews	227575	Teeberg	PR_2013	-33.3292	22.04331
compacta	R. Br.	227564	Kleinmond_1	PR_2032	-34.3307	19.0269
comptonii	Beard	245134	Songmivelo_1	PR_2049	-25.7873	31.08299
cordata	Thunb.	230152	Garcia_Pass_4	PR_2021	-33.9574	21.19069
cordata	Thunb.	230152	Kogelberg_3	PR_2028	-34.3112	18.94826
coronata	Lam.	230373	Potberg_Trail_1	PR_2003	-34.3774	20.57179
coronata	Lam.	227581	Bergfontein_1	PR_2022	-34.0214	21.45853
cryophila	Bolus	228689	Sneeuberg_gully_upper	PR_2038	-32.5118	19.1561
curvata	N.E. Br.	248060	Diggers_Rest	PR_2050	-25.6686	31.0459
cynaroides	(L.) L.	230370	Potberg_Trail_3	PR_2006	-34.3674	20.54655

cynaroides	(L.) L.	227574	Garcia_Pass_3	PR_2020	-33.9562	21.18967
cynaroides	(L.) L.	227579	Kogelberg_2	PR_2027	-34.309	18.94571
decurrens	E. Phillips	230364	Bergfontein_2	PR_2023	-34.0384	21.41108
denticulata	Rourke	230371	Potberg_Trail_2	PR_2004	-34.3763	20.57062
dracomontana	Beard	248056	Royal_Natal_2	PR_2053	-28.677	28.92834
dracomontana	Beard	248059	Royal_Natal_3	PR_2054	-28.7308	28.91215
eximia	(Salisb. ex Knight) Fourc.	230303	Baviaanskloof_1	PR_2007	-33.697	24.11971
eximia	(Salisb. ex Knight) Fourc.	227582	Garcia_Pass_1	PR_2018	-33.9572	21.21965
gaguedi	J.F. Gmel.	248057	Blyde_River_3	PR_2045	-24.5507	30.76981
gaguedi	J.F. Gmel.	248040	Songmivelo_1	PR_2049	-25.7873	31.08299
glabra	Thunb.	227591	Pakhuis_Pass	PR_2040	-32.1354	18.9916
glabra	Thunb.	255946	Oorlogskloof2	PR_3002	-31.37300	19.04115
grandiceps	Tratt.	230363	Garcia_Pass_3	PR_2020	-33.9562	21.18967
intonsa	Rourke	230301	Baviaanskloof_4	PR_2010	-33.5235	24.08932
intonsa	Rourke	256140	Blesberg_3	PR_2063	-33.4153	22.689
lacticolor	Salisb.	141623	GW	GW	-34.1195	18.97258
lacticolor	Salisb.	141485	LI	LI	-33.6951	19.13143
lacticolor	Salisb.	141479	LK	LK	-34.0394	18.99126
lacticolor	Salisb.	141486	PK	PK	-34.0183	19.07807
lacticolor	Salisb.	141480	PO2	PO2	-33.9676	19.14433
laetans	L.E. Davidson	248039	Blyde_River_4	PR_2046	-24.5723	30.77986
lanceolata	E. Mey. ex Meisn.	230375	Noetsie	PR_2001	-34.4412	20.73684
laurifolia	Thunb.	227577	Middleberg	PR_2034	-32.6357	19.15701
laurifolia	Thunb.	227584	Pakhuis_Pass	PR_2040	-32.1354	18.9916
laurifolia	Thunb.	256127	Bains_Kloof2	PR_2060	-33.6293	19.10114
laurifolia	Thunb.	255951	Oorlogskloof2	PR_3003	-31.37300	19.04115
lepidocarpodendron	(L.) L.	230361	Kleinmond_2	PR_2033	-34.3353	19.01525
lepidocarpodendron	(L.) L.	255944	Kalk_Bay	PR_2058	-34.1213	18.44649
longifolia	Andrews	266419	Houw_Hoek_3	PR_2031	-34.2119	19.16504
lorifolia	(Salisb. ex Knight) Fourc.	228476	Baviaanskloof_3	PR_2009	-33.5222	24.08711
lorifolia	(Salisb. ex Knight) Fourc.	227567	Seweweekspoort_2	PR_2017	-33.3782	21.3138

lorifolia	(Salisb. ex Knight) Fourc.	227569	Garcia_Pass_5	PR_2024	-33.931	21.20104
magnifica	Andrews	227599	Sneeuberg_gully_upper	PR_2038	-32.5118	19.1561
montana	E. Mey. ex Meisn.	230155	Waboomberg_1	PR_2014	-33.3524	22.03298
montana	E. Mey. ex Meisn.	256141	Blesberg_2	PR_2062	-33.4176	22.68694
mundii	Klotzsch	141617	KM	KM	-34.3272	19.0027
mundii	Klotzsch	141616	MS	MS	-34.3883	19.35095
mundii	Klotzsch	141620	OB	OB	-34.334	18.93185
mundiieast	Klotzsch	230304	Baviaanskloof_1	PR_2007	-33.697	24.11971
mundiieast	Klotzsch	141619	BS	BS	-33.6097	24.50043
mundiieast	Klotzsch	141618	TK	TK	-33.9414	24.18693
nana	(P.J. Bergius) Thunb.	256129	Bains_Kloof	PR_2059	-33.6286	19.10092
neriifolia	R. Br.	230306	Potberg_Road_1	PR_2002	-34.4317	20.70683
neriifolia	R. Br.	230153	Garcia_Pass_1	PR_2018	-33.9572	21.21965
nitida	Mill.	230298	Swartberg_1	PR_2012	-33.3678	22.10204
nitida	Mill.	227570	Garcia_Pass_6	PR_2025	-33.9495	21.22878
nitida	Mill.	228738	Houw_Hoek_2	PR_2030	-34.2094	19.17525
nitida	Mill.	227588	Pakhuis_Pass	PR_2040	-32.1354	18.9916
nitida	Mill.	227587	Uitkyk_Pass_2	PR_2042	-32.4068	19.10799
nitida	Mill.	255948	VR_Pass	PR_3000	-31.37244	19.04123
nubigena	Rourke	248038	Royal_Natal_4	PR_2055	-28.7373	28.90165
odorata	Thunb.	256132	Malmsbury	PR_2065	-33.7622	18.76767
parvula	Beard	248054	Long_Tom_Pass_2	PR_2047	-25.1435	30.62235
piscina	Rourke	227578	Middleberg	PR_2034	-32.6357	19.15701
piscina	Rourke	255939	Tradouws_Pass	PR_2057	-33.9861	20.72102
pruinosa	Rourke	256133	Blesberg_2	PR_2062	-33.4176	22.68694
punctata	Meisn.	230299	Baviaanskloof_5	PR_2011	-33.5228	24.08797
punctata	Meisn.	227573	Teeberg	PR_2013	-33.3292	22.04331
punctata	Meisn.	227576	Sneeuberg_gully_upper	PR_2038	-32.5118	19.1561
punctata	Meisn.	141608	BBP	BBP	-33.4193	22.68854
punctata	Meisn.	141614	CB	CB	-32.5126	19.18215
punctata	Meisn.	141615	GB	GB	-33.254	19.48446

punctata	Meisn.		JK	JK	-33.9695	19.5017
punctata	Meisn.	141621	KS	KS	-33.6451	22.95381
punctata	Meisn.	141613	SPP	SPP	-33.6451	22.95381
recondita	H. Buek ex Meisn.	227598	Sneeuberg_gully_1	PR_2037	-32.5109	19.1581
repens	(L.) L.	230374	Potberg_Road_1	PR_2002	-34.4317	20.70683
repens	(L.) L.	230300	Baviaanskloof_5	PR_2011	-33.5228	24.08797
repens	(L.) L.	230297	Swartberg_1	PR_2012	-33.3678	22.10204
repens	(L.) L.	228490	Garcia_Pass_2	PR_2019	-33.9679	21.2198
repens	(L.) L.	227580	Houw_Hoek_3	PR_2031	-34.2119	19.16504
repens	(L.) L.	228489	Kleinmond_1	PR_2032	-34.3307	19.0269
repens	(L.) L.	228484	Uitkyk_Pass_1	PR_2041	-32.4068	19.10799
repens	(L.) L.	256128	Bains_Kloof2	PR_2060	-33.6293	19.10114
repens	(L.) L.	255947	Oorlogskloof	PR_3001	-31.37547	19.04776
roupelliae	Meisn.	245139	Long_Tom_Pass_2	PR_2046	-25.1435	30.62235
roupelliae	Meisn.	245139	Long_Tom_Pass_2	PR_2047	-25.1435	30.62235
roupelliae	Meisn.	248055	Songmivelo_2	PR_2051	-25.795	31.08337
rubropilosa	Beard	248041	Blyde_River_2	PR_2044	-24.5769	30.79812
rupicola	Mund ex Meisn.	256137	Blesberg_1	PR_2061	-33.4191	22.68793
scabra	R. Br.	227593	Kogelberg_1	PR_2026	-34.3199	18.96021
scabra	R. Br.	227571	Houw_Hoek_2	PR_2030	-34.2094	19.17525
scolopendriifolia	(Salisb. ex Knight) Rourke	230154	Waboomberg_2	PR_2015	-33.3531	22.033
scolopendriifolia	(Salisb. ex Knight) Rourke	227583	Sneeuberg_Hut_2	PR_2036	-32.4884	19.15175
scolopendriifolia	(Salisb. ex Knight) Rourke	256130	Blesberg_4	PR_2064	-33.4204	22.69119
speciosa	(L.) L.	227572	Garcia_Pass_3	PR_2020	-33.9562	21.18967
subulifolia	(Salisb. ex Knight) Rourke	227566	Houw_Hoek_1	PR_2029	-34.2007	19.15497
subvestita	N.E. Br.	248042	Royal_Natal_1	PR_2052	-28.6859	28.9156
subvestita	N.E. Br.	141612	SB1	SB1	-32.6939	25.58685
subvestita	N.E. Br.	141610	SB2	SB2	-32.5805	26.93345
subvestita	N.E. Br.	141609	SB3	SB3	-30.9236	28.20812
subvestita	N.E. Br.	141504	SB4	SB4	-30.5454	29.6563
subvestita	N.E. Br.	141503	SB5	SB5	-29.6093	29.36219

subvestita	N.E. Br.	141502	SB6	SB6	-28.6836	28.91817
sulphurea	E. Phillips	230362	Seweweekspoort_1	PR_2016	-33.3765	21.3147
susannae	E. Phillips	230372	Lekkerwater	PR_2005	-34.4273	20.64887
tenax	(Salisb.) R. Br.	230302	Baviaanskloof_2	PR_2008	-33.693	24.13613
venusta	Compton	227596	Waboomberg_1	PR_2014	-33.3524	22.03298
venusta	Compton	141500	BBV	BBV	-33.4186	22.68702
venusta	Compton	141505	KSV	KSV	-33.6489	22.78194
venusta	Compton	141611	MJV	MJV	-33.6211	22.91521
venusta	Compton	141607	MJV2	MJV2	-33.3532	22.0503
venusta	Compton	141866	SPV	SPV	-33.3469	22.09973
venusta	Compton	141606	WB	WB	-33.3524	22.03304
welwitschii	Engl.	245138	Mashsishing	PR_2048	-25.1459	30.30664
witzenbergiana	E. Phillips	227589	Sneeuberg_Hut_1	PR_2035	-32.4891	19.15159

Chapter 4: Causes of microscale trait-environment associations in two closely related South African shrubs

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Abstract

Relationships between plant traits and environmental variables on a global scale have often been interpreted as representing fundamental ties between plant strategies and their climatic constraints. The extent to which these global patterns arise from patterns at the local, microgeographic scale, and whether the same processes are responsible for local and global associations remains to be determined. Here, I examine trait associations at the microenvironmental scale in two species (*Protea punctata* and *P. venusta*) from a diverse plant radiation in a heterogeneous site within the Cape Floristic Region of South Africa, a biodiversity hotspot. Furthermore, I use a controlled greenhouse dry-down experiment to determine whether detectable associations are the result of phenotypic plasticity or genetically-based environmental filtering associated with differential establishment. I detected relationships in the field within species at the scale of a few hundred meters, but the relationships differed between the two species. These field-based associations were not replicated in greenhouse-grown seedlings, suggesting that plasticity may be responsible for relationships detected in the field. However, I also found that seedlings from higher elevations in both species perform better overall than those from lower elevations, although relative performance in dry-down vs control treatments was not related to maternal location.

Introduction

Plants have evolved to closely match the environments in which they are found, and often exhibit similar forms in similar environments in evolutionarily and geographically independent lineages. Examples of broad convergence, indicative of macro-scale adaptive processes, include the evolution of succulent life forms in desert environments in Euphorbiaceae in the Old World and Cactaceae in the New World, and convergent use of pitcher-like structures as carnivorous structures found in low-nutrient environments in *Nepenthes* and *Sarracenia*. These striking examples are the result of millions of years of evolution in response to extreme environments. Do local processes result in similar trait-environment relationships at smaller geographic and temporal scales?

Broad correlations between overall form and environment are observed in specific plant traits and climatic variables. Globally, the highly cited worldwide leaf economics spectrum (LES) and its environmental correlates suggest that resource acquisitive traits are associated with lower temperatures and more rainfall, while the opposite is true for resource conservative traits (Wright et al. 2004; Wright et al. 2005). Numerous other studies have found similar patterns related to a variety of traits at this scale (e.g. Reich et al. 1999; Moles et al. 2009; Moles et al. 2014). Although global generalizations across taxa are valuable, they also leave large amounts of variation in trait values unexplained, and they may not reflect the evolutionary processes that are responsible for that variation (Mason and Donovan 2015; Mason et al. 2016). Worldwide patterns may reflect environmental filtering of pre-existing trait variation rather than an accumulation of differences among populations within species at different scales. As a result, patterns may differ among hierarchical levels within a clade, across different geographical scales, or both (Simpson's paradox, Simpson 1951). Trait-environment patterns may differ across regions with similar biomes (Forrestel et al. 2017), within regions (Cavender-Bares et al. 2004; Mitchell et al. 2015), among species within communities (Lajoie and Vellend 2015), or be genus-specific (Cavender-Bares et al. 2004; Mason and Donovan 2015). If patterns are inconsistent across scales, the processes or causes of associations are likely to be different, as are their consequences.

CAUSES OF LOCAL TRAIT-ENVIRONMENT RELATIONSHIPS

Evolution occurs at the population level, but the extent to which global patterns of trait-environment relationships are detectable at the microenvironmental level and the causes of such associations are not well-studied, except in extreme cases. The exceptions are primarily examples of local adaptation in herbaceous perennials, as with heavy metal tolerance on mine tailings and roadsides (Jain and Bradshaw 1966; Antonovics and Bradshaw 1968) and adaptation of ecotypes to mown vs unmown habitats (McLeod et al. 2012). Adaptation along less extreme gradients is not as well characterized, or has not been detected (e.g. Becker et al. 2006)). Local (within-population) trait-environment associations could reflect heritable trait differences or non-heritable plastic responses to the environment. Local adaptation could produce these patterns if variation associated with the environment is heritable and there are barriers to gene flow across sites. At a local scale, gene flow is expected to swamp the effects of differential stabilizing selection across genotypes (Slatkin 1973; Lenormand 2002) unless there is extremely strong selection with a very strong gradient or turnover in habitat (Linhart and Grant 1996).

Heritable trait differences could underlie local trait-environment associations either because limited dispersal and natural selection result in *in situ* adaptive evolution or because environmental filtering of pre-existing variation results in sorting of genotypes along a gradient within a site (Kraft et al. 2015). In this case, trait-environment associations reflect filtering as a means of within-generation selection, not evolution by natural selection. Climatic gradients are less likely to be as sharply defined, as mine tailings in plants or along the rocky shore in marine snails (Johannesson et al. 1995), for example, so I expect filtering rather than *in situ* adaptation., In plants, the consequences of filtering are more apparent at later life stages than at earlier stages, suggesting that filters are actually acting at early or intermediate life stages (Yang et al. 2016). Species or genotypes that cannot survive a specific condition are likely to be "filtered" out early on. In particular, in areas prone to water limitation during all or parts of the year, this may be associated with survival during dry periods (subject to water stress), and precipitation is a larger factor in driving natural selection across the globe than even temperature (Siepielski et al. 2017). If

heritable variation underlies local trait-environment associations in plants, it may often be associated with this environmental filtering at the seedling stage, rather than *in situ* adaptation.

Of course, local trait-environment associations might also reflect plastic responses to environmental variation at the local scale. Although the pattern and extent of phenotypic plasticity is heritable, differences in traits attributed to plasticity are phenotypic differences associated with a single genotype in different environments, i.e., differences in position along a phenotypic reaction norm (Schlichting 1986). If trait-environment associations observed in the field are not observed in controlled conditions, phenotypic plasticity might be the cause of these relationships. The relative contribution of environmental filtering and plasticity to trait-environmental associations at a local population scale remain to be determined.

STUDY SYSTEM

I test for trait-environment relationships at a local population scale within an evolutionary radiation in an environmentally heterogeneous region of the world, the Cape Floristic Region (CFR) of South Africa. This "biodiversity hotspot" is home to over 9000 plant species, ~70% of which are endemic (Rourke 1982; Goldblatt and Manning 2000; Myers et al. 2000). The CFR is topographically, edaphically, and climatically complex, with multiple gradients in rainfall amount and seasonality, temperature, and fire regime (Linder and Hardy 2004; Linder 2005; Linder 2014). Moreover, much of the diversity in the CFR is held within radiations of only 33 plant lineages (Linder 2003; Linder and Hardy 2004; Schnitzler et al. 2011), one of which is the genus *Protea* (L.), Proteaceae. *Protea* is a diverse genus of 112 species, all of which are found on the African continent, and its center of diversity is in the CFR. The crown age of the genus is estimated at 5 – 18 million years, and there are radiations within the genus, such as the white proteas, which diversified rapidly within the past 0.34 – 1.2 million years (Sauquet et al. 2009; Prunier and Holsinger 2010; Valente et al. 2010).

Previous work has found evidence for genus-wide, clade level, and intraspecific trait-environment associations with varied responses to drought stress (Carlson et al. 2011; Prunier et al. 2012; Heschel et

al. 2014; Mitchell et al. 2015; Mitchell et al. Under Review). Here I ask whether similar associations can be detected within a population, i.e., at a microgeographic scale. The populations I study occur at a single site with a steep gradient in elevation. I combine the association between elevation, aspect, and insolation with daily temperature and humidity measurements to assess trait-environment associations. The site is home to two closely related, but morphologically distinct, *Protea* species within the well-supported white protea clade, *P. punctata* and *P. venusta*. *Protea punctata* is a broadly-distributed erect shrub approximately two meters tall with relatively large leaves, while *P. venusta* is a geographically-restricted low sprawling shrub 0.7 meters tall with relatively small leaves (Figure 1) (Rourke 1982; Rebelo 2001; Valente et al. 2010; Mitchell et al. 2017). In spite of the significant morphological differences, both morphological and genetic hybrids between them have been detected at this site (Prunier and Latimer 2010; McIntosh et al. 2014). I measured individual plants in the field and seedling offspring in a controlled water stress experiment in the greenhouse to ask:

1. ***Are trait-environment relationships detectable at the microenvironmental scale?*** If so, relationships are expected to be the same in closely-related species, and similar to global trends.
2. ***Are detectable trait-environment associations the result of genetic differentiation or the result of phenotypic plasticity?*** If greenhouse seedling trait values do not match field values, plasticity must underlie trait-environment associations in the field. If seedlings exhibit differential relative survival and performance under stress related to maternal home environment, environmental filtering may be contributing to observed trait-environment patterns.

Methods

FIELD SAMPLING

I haphazardly sampled *Protea punctata* and *Protea venusta* individuals (86 and 61 individuals, respectively) on Blesberg Mountain (approximately -33.52° latitude, 22.69° longitude, peak elevation

~2000 m) in the Swartberg Mountain range, Western Cape, South Africa in late May 2013 (Figure 1). For each individual, I recorded latitude, longitude, and elevation using a Garmin eTrex Vista HCx GPS (Olathe, Kansas). The greatest distance between samples was approximately 650 meters, but most plants were within 300 meters of each other. I collected a fully-expanded leaf and a wood sample subtending the current year's growth, measured plant height and canopy dimensions, and counted the number of seedheads for each plant. I measured leaf fresh and dry weight, and scanned images of the leaves to calculate leaf area, leaf length, and leaf width using ImageJ. I used clear nail varnish and tape to make stomatal peels that were later analyzed under a light microscope to estimate stomatal density. Wood density was estimated using a water displacement method as dry mass/wet volume.

I collected 1-3 seedheads from 64 "mother" plants (39 *P. punctata,* 22 *P. venusta,* and 3 "hybrids") from the mountainside. I dried them, allowing the heads to open up and seeds to be easily picked. I sorted the seeds (discarding any non-viable individuals) and took the average weight of seeds from each individual seedhead. Additional seeds were collected in August 2014. For a subset of the mothers (18 *P. punctata*, 6 *P. venusta*, and 3 "hybrids"), leaf stable isotopes were analyzed at the Center for Stable Isotope Biogeochemistry, Department of Integrative Biology, University of California, Berkeley.

FIELD ENVIRONMENTAL DATA

I used the ASTER Global Digital Elevation Map (GDEM; NASA Land Processes Distributed Active Archive Center, 30 –m grid) to extraction elevation (m) and to calculate aspect (in radians, where 0 is directly east) of each plant location using the terrain function in the "raster" package in R (R Core Team 2010; Hijmans and van Etten 2014). To estimate insolation for each individual point, I used the ArcGIS Spatial Analyst extension (ESRI 2014), projecting the GDEM onto the coordinate system WGS 1984 UTMZone 34S. I estimated insolation for a full year (2013) with monthly intervals, a sky/size resolution of 200, and default values for the remaining parameters.

I selected 10 "mother" individuals (five of each species) spatially distributed across the mountainside as sites for microenvironment data collection (Figure 1). I attached Haxo-8 data-loggers (LogTag Recorders, Auckland, New Zealand) to the selected plants and collected data on temperature and humidity every 66 minutes for a full year (May 2013 – May 2014). Data-loggers were retrieved in August 2014 and I calculated summary statistics for each site, including average, minimum, maximum, and seasonal average temperatures and humidity measures. Soil samples from each data-logger site were analyzed for nutrient content at Bemlab (Cape Town, South Africa).

DRY-DOWN EXPERIMENT

I ran a dry-down experiment on seedlings derived from field-collected seed to assess both trait-environment relationships in a controlled setting and differential response to water stress. Prior to germination, seeds were surface sterilized in 1% bleach for 5 minutes, then rinsed 3 times with double distilled water. Seeds were planted in 288 plug flats filled with standard #2 nursery mix (Company, Location) and placed in a Conviron CMP4030 growth chamber (Winnipeg, Canada) with short days (10H, 20°C) and cool nights (14H, 8°C) to simulate winter conditions in South Africa. 373 *Protea punctata* seeds from 20 maternal lines were sown in September 2015. Germination trials showed a slower germination rate for *P. venusta*. To more closely match initial seedling sizes, 347 *P. venusta* seeds from 13 maternal lines were sown in August 2015. Germination was assessed every 1 – 2 days, with germination marked as the first emergence of the hypocotyl above the soil surface.

456 germinated seedlings were chosen for the dry-down experiment. Seedlings from each maternal line were randomly assigned to either drought or control treatments. Seedlings were randomly assigned to large plastic trash bins (19 pots per bin) and within each bin haphazardly transplanted into 4" x 30" tall tree pots (Stuewe & Sons, Tangent, Oregon) filled with a mix of 5:4:2:1 parts peat moss, sand, perlite, charcoal, respectively, in September 2015. Seedlings were watered every 2-3 days for 14 weeks after transplanting and plant height (to approximately the apical meristem) was measured weekly.

Plants were watered well on February 14, 2016 and the dry-down began February 15. I selected a subset of 144 plants, ensuring that an individual from each bin and from each maternal line was chosen, for measurement of stomatal conductance and volumetric water content of the pots throughout the dry-down. Stomatal conductance measures were performed mid-day (between 10:00 and 14:30) using a leaf porometer, model SC-1 (Decagon Devices, Pullman, WA). Volumetric water content (VWC) was measured using a HydroSense II soil-water sensor using the CS658 20cm water content probe (Campbell Scientific, Logan, UT). Plants within barrels assigned to the control treatment were watered every 2-3 days, while those in the drought treatment received no water for nine weeks.

The dry-down ended April 18, 2016 after nine weeks, based on signs of plants shutting down as assessed by drops in stomatal conductance. I measured stomatal conductance and volumetric water content for all individuals harvested seedlings and measured plant height, harvested a single most-recently expanded leaf per plant and scanned it for leaf area, and measured the mass of this leaf as well as the entire fresh weight of the aboveground biomass. I made stomatal peels for the abaxial side of each trait-selected leaf using clear nail varnish and used these to measure stomatal density and size. After the above-ground harvest, I sliced open the tree pots and gently extracted the roots and rinsed them free of soil, taking care not to damage or lose the fine roots. Shoots, roots, and the selected leaf were dried in a 60°C oven for two weeks and then weighed. I measured leaf areas, lengths, and widths from the leaf scans in the program ImageJ. Stomatal peels were analyzed under a light microscope at 40x magnification, with stomatal densities averaged across three viewing fields.

FIELD TRAIT – ENVIRONMENT ANALYSES

For the greenhouse experiment, I asked the following questions: 1) Are there detectable trait-microenvironment relationships? 1) Are detectable relationships the same or different between the two focal species?

To detect field trait-environment relationships, I repeatedly analyzed a single trait with all environmental covariates at once with separate slopes and intercepts for each species. My dataset includes

a large number of trait and environmental variables. For the environment, I chose variables based on a correlation heatmap built using the heatmap() function in R for my 10 observed environmental microsites (Fig. S1). I chose variables commonly used in the literature from each major cluster of the dendrograms: average relative humidity (RH, %), average temperature (AVGT, °C), maximum temperature (MAXT, °C), available soil phosphorous measured using Bray II (SOILP mg/kg), and soil pH measured using KCl (SOILpH) (Table 1, Table S1). I chose to analyze traits commonly used in the literature and used in previous genus-wide evolutionary analyses in *Protea*: canopy area (CANOPY), leaf carbon-to-nitrogen ratio (CNRATIO), leaf $\delta^{13}C$ (D13C) leaf fresh water content (FWC), plant height (HEIGHT), leaf area (LA), leaf length-to-width ratio (LWR), leaf mass per area (LMA), leaf nitrogen per mass (NMASS), stomatal density (SD), and wood density (WOOD) (Table 1, Table S2).

I built Bayesian models in JAGS (Plummer 2003) run through R using the package R2jags (Su and Yajima 2009) that estimate the relationship between location and observed environmental covariates at the 10 microsites and uses that relationship to impute environmental covariates at the 137 plant sites where these were not directly measured. The models simultaneously use the measured and imputed covariates to assess the relationship between an individual trait and all environmental covariates using separate slopes and intercepts for *P. punctata* and *P. venusta* (Figure 2). By combining the imputation model and the environmental regression model, I account for uncertainty in the imputation by imputing multiple values for the covariates in proportion to their weight in the posterior density. My JAGS settings included 5 chains, a burn-in of 5000, 25000 iterations, and posterior sample thinning every 25 iterations. Models were checked for convergence, insuring that Rhat values were 1.01 or less, and visually assessed using traceplots. I calculated modified R^2 values according to Gelman and Pardoe (2006) for each species and overall. To assess the reliability of point estimates I computed symmetric 95% and 80% credible intervals from the posterior distributions. To compare coefficients between *P. punctata* and *P. venusta*, I used all posterior samples and determined if the species had relationships in the same or different direction, resulting in posterior estimates for these as well.

GREENHOUSE ANALYSES

For the greenhouse experiment, I asked the following questions: 1) Can field trait-environment relationships in mature plants be detected in a controlled setting at the seedling stage? 2) Do trait values exhibit plasticity in response to dry-down stress? 3) Is performance during water stress related to position is the field?

Are offspring traits related to maternal position?

To determine if trait-environment relationships at the microscale are genetically influenced, I asked if seedling traits are associated with the environmental location of their mother. I used the same modeling approach for these data as for the field data, and I included a random intercept term for treatment (dry-down vs control). For this analysis, I increased the burn-in to 25000 and total iterations to 75000 to obtain a large enough effective sample size for the treatment effect to approach Rhat values near 1.00. I also calculated adjusted R^2 values, constructed 95% and 80% credible intervals, and compared the signs of species' relationships and treatment effect values from the posterior distributions. Additionally, I sub-sampled 4000 samples from the posteriors of each species greenhouse trait-environment relationship to compare with the field trait-environment relationships.

Do traits exhibit plasticity in response to stress?

I estimated plasticity in response to dry-down stress as the slope of the reaction norm between treatments in the greenhouse. I built separate models for each trait in JAGS, with trait values modeled as a response to treatment with separate values for each species and included random effects for maternal line and bin to account for half sibling families and position in the greenhouse, respectively. My JAGS settings for these analyses were 5 chains, a burn-in of 5000, 25000 iterations, and posterior sample thinning every 25 iterations. I also calculated adjusted R^2 values, constructed credible intervals, and compared species slopes as above.

Is stress performance related to home microenvironment?

I estimated survival in dry-down seedlings relative to control seedlings. To do this, I built a model in JAGS with status (alive or dead) modeled as a Bernoulli random variable with the probability of survival modeled as a logit-transformed function of maternal line, bin in the greenhouse, and relative survival. The model was allowed a 5000 iteration burn-in followed by 75000 iterations, thinned every 75, and run on five chains. As measures of performance, I modeled total biomass (root_biomass + shoot_biomass) as a function of maternal line, bin, and relative performance, and total biomass as a function of root:shoot ratio (RSratio, root_biomass / shoot_biomass) to determine relationships between performance and particular drought traits. I estimated heritability for survival, performance, and RSratio from the standard deviations among mothers and among individuals.

Results

FIELD RELATIONSHIPS

Is there microenvironmental variation associated with position?

There was substantial microenvironmental variation within my field site associated with position along the 288 meter elevation gradient, aspect, or insolation (Table 2). Average temperature was negatively associated with elevation and positively associated with insolation and ranges 2.9°C across 10 microsites. Extremes appear to be important, as the maximum temperature (MAXT) ranged 13.6°C and had a strong positive association with insolation. Belowground, soil pH ranged 1.3 units and was negatively associated with both elevation and aspect (Table 2, Table S1, Table S2).

Trait – microenvironment relationships

I identified trait-environment associations in both species, but none of the well-supported associations were shared. Overall, I found strong evidence (95% credible interval not overlapping zero) for two associations, both in *P. punctata*, and moderate evidence (80% credible interval not overlapping

zero) for six additional associations (Figure 3, Table S3). In *P. punctata*, bigger plants (in terms of both HEIGHT and CANOPY) are associated with higher maximum temperature, and smaller leaves are associated with higher elevations. Denser wood is associated with more basic soil, and fleshier leaves (higher FWC) are associated with more available phosphorus (SOILP). In *P. venusta*, less succulent, more sclerophyllous leaves with more densely packed stomates are associated with higher maximum temperatures (Figure 3, Table S3).

I detected no sign differences between *P. punctata* and *P. venusta* for any of the trait-environment relationships I studied (Table S3), but my ability to detect differences was limited by the broad credible intervals associated with each coefficient. Based on posterior comparisons of the sign of relationships between species, I found moderate support (80% credible interval did not overlap zero) for associations in the same direction in only two relationships. LMA is positively associated with MAXT and FWC is positively associated with SOILP (detected in *P. punctata*) in both species, although only the FWC-SOILP relationship in *P. punctata* is strongly supported as positive. The highest posterior support is for associations to be in the same direction for all but two relationships. LEAFAREA and ELEV (negative in *P. punctata*, posterior support for differing = 0.676) and CANOPY and MAXT (positive in *P. punctata*, posterior support for differing = 0.557) had higher support for the relationships differing in sign across species, but not significantly.

GREENHOUSE RELATIONSHIPS

Seedling trait-environment relationships and plasticity

Despite dry-down pots having extremely low water content (VWC of 1.2%), plants at the end of the dry-down were just beginning to shut down with respect to stomatal conductance. Average volumetric water content of the control plants was about 10.8% and for the dry-down plants was approximately 1.2%, with corresponding conductances of 135.2 mmol $\cdot$ $m^{-2} \cdot s^{-1}$ in *P. punctata* and 142.1 in *P. venusta* in the control, and 120.4 and 90.4 in the dry-down, respectively (Table 3). Surprisingly, survivorship was

higher in the dry-down treatment, and dry-down plants were also larger on average than control plants, though there was more variability in dry-down plants (Table 3).

I was unable to detect any associations between seedling traits in the greenhouse and maternal environments in the field except for a negative association between seedling height in *P. punctata* and maximum temperature experienced by the mother (Figure 4, Table S4). This single detected relationship is in the same direction as in the field, but may be a measure of overall health at the seedling stage rather than a predictor of adult height. I did not find any cases in which the sign of the estimated relationships strongly matched or differed from the relationships in the field, including ones where there was a detectable relationship in maternal plants (Table S4). R-squared values for these models were generally quite low compared with the field trait-environment relationships (Table S5). The failure to detect any pattern may reflect a lack of statistical power: 20 maternal lines of *P. punctata* and 13 maternal lines of *P. venusta* were available for the experiment.

In the greenhouse experiment, I was only able to detect plasticity between the two treatments in leaf area and performance (combined root and shoot biomass) in *P. punctata*, and no significant plasticity in *P. venusta* (Figure 5, Table S6). Leaves and seedlings overall unexpectedly were bigger under dry-down stress than in the control treatment, and there is good evidence that the overall performance difference is in the same direction in both species. Other functional traits did not exhibit plasticity as measured by the slope of the reaction norm.

Seedling survival and performance

My experimental results at the seedling stage are inconsistent with my expectations from the field. Higher elevations are associated with cooler temperatures and higher humidity, while lower elevations experience warmer temperatures and lower humidity, which I expect to reflect higher water stress. If trait differences were genetic, I expected seedlings from lower elevations to do relatively better in the dry-down than plants from higher elevations.

I detected no differences in survival as a function of maternal elevation in either *P. punctata* or *P. venusta* (Table 4). Similarly, performance of dry-down plants relative to control plants (measured as total biomass) is unrelated to the location of the mother (Figure 6A). There is a weakly supported trend for performance to be better in seedlings from higher elevations, but there is no support for differences in relative performance among treatments associated with position or with species or treatment. Performance does appear to have a heritable component, though there is substantial variation among individuals and within maternal lines (Table S7). My measure of performance in the greenhouse could be related to root:shoot ratio. Relative RSratios between dry-down and control individuals have no association with elevation, but seedlings derived from higher elevations overall have higher RSratios, just as seedlings from higher elevations have higher performance (Figure 6B). Interestingly, the drought-related trait RSratio has a lower heritability than overall performance (Table S5).

Discussion

THE SCALE OF TRAIT-ENVIRONMENT RELATIONSHIPS

Trait-environment relationships are often presumed to be based on fundamental tradeoffs associated with a "fast-slow" continuum of growth strategies and environmental conditions (Wright et al. 2004; Wright et al. 2005). However, patterns of trait-trait integration are often not the same at different ecological scales because the processes influencing variation differ across scales (Messier et al. 2010; Messier et al. 2016). The same is likely true for trait-environment associations. I asked if trait-environment relationships are detectable at the microenvironmental level. Across a gradient spanning only a few hundred meters, I found evidence for several associations within species related to position, temperature, and soil characteristics (Figure 3). Although the study region is small, it spans a steep elevation gradient (288 meters in elevational difference) which likely mediates much of the environmental variation.

Associations at this scale are not unheard of, and may be related to both inter- and intraspecific variation (Auger and Shipley 2013). Ackerly (2002) detected relationships between specific leaf area (SLA, the inverse of LMA) and overall leaf area both within species and communities associated with elevation, aspect, and insolation in California, while Mitchell et al. (2016) detected variation in traits along a temperate wet-dry ecotone. On the other hand, within the CFR, trait-environment relationships are rarely detectable at the community-level (Aiello-Lammens et al. 2016).

(IN)CONSISTENCY OF RELATIONSHIPS IN *PROTEA*

If trait-environment relationships are universal, closely related species with similar life histories ought to relate to their environments in similar ways, but the extent to which traits exhibit predictive relationships with environmental gradients is still not well characterized (Shipley et al. 2016). However, the same functional relationship does not mean that worldwide patterns are necessarily a simple extrapolation or accumulation of population-level differentiation. Similar patterns may still reflect different causes, and if patterns are different, this implies that the processes generating these patterns differ across scales. In this case, I studied trait-environment relationships on a small geographical scale in two recently diverged species, and I detected several differences in trait-environment relationships (Figure 3). The sprawling shrub, *P. venusta*, exhibits traits toward the "slow" end of the spectrum when found in hotter areas. The taller shrub, *P. punctata*, has a more ambiguous strategy; it is larger overall in hotter areas with larger leaves at lower elevations. These species thus are relating differently to their microenvironment.

Unexplained variation in functional traits has been attributed to a diversity of species-specific strategies (Westoby et al. 2000; Poorter et al. 2009; Lajoie and Vellend 2015). Proteaceae in Australia exhibit diversity in leaf shapes that presumably reflect the range of solutions to different functional tradeoffs (Nicotra et al. 2011), and *Protea* species in South Africa may also employ diverse strategies. There is evidence for overdispersion of traits within communities of *Protea* and their relatives, though not necessarily more than expected by chance (Cody 1986; Potts et al. 2011), but at this scale there is a

tendency for closely related *Protea* species to co-occur more often than expected (Jiang et al. 2013). I find that morphologies are different between focal species, and traits appear to relate to their environments in unique ways as well. Communities are often overdispersed in terms of traits at narrow taxonomic scales (Cavender-Bares et al. 2006), consistent with our findings in two coexisting, closely related species of *Protea*.

The fact that individual species do not respond in the same way may be one explanation for the lack of trait-environment associations detected in this region at the community-level (Aiello-Lammens et al. 2016): species within a community have traits that relate to their environment in different ways, resulting in a lack of signal, *a la* Simpson's paradox (Simpson 1951).

We have previously found evidence for trait-environment relationships in *Protea* within a species (Carlson et al. 2015), within a subclade (Carlson et al. 2011; Prunier et al. 2012), and across the genus and at both contemporary and evolutionary time scales (Mitchell et al. 2015), Mitchell et al. (Under Review). In this study, I find that such patterns do not necessarily hold at lower taxonomic levels or smaller spatial scales, consistent with detection of different patterns both among genera within the region (Mitchell et al. 2015) and among species within the white proteas (Prunier et al. 2012). Some consistencies across scales include *P. punctata* being larger at higher temperatures and having smaller leaves at higher elevations (Prunier et al. 2012; Mitchell et al. 2015; Mitchell et al. Under Review).

Across populations, Prunier et al. (2012) found no trait-environment associations in *P. venusta*, although I detected them here at a much smaller scale. The finding of more sclerophyllous leaves (higher LMA) at high temperatures is consistent with subclade-level patterns and genus-wide contemporary patterns (Carlson et al. 2011; Mitchell et al. 2015), but not necessarily evolutionary patterns, where LMA is more tightly linked to rainfall (Mitchell et al. Under Review). Finally, higher stomatal density at high temperatures in *P. venusta* is consistent with patterns across populations of the broadly distributed species *P. repens* as well as contemporary (but not evolutionary) genus-wide patterns (Carlson et al. 2015; Mitchell et al. Under Review).

INTERPRETATION OF RELATIONSHIPS

Local trait-environment relationships in *Protea* are varied, but detected relationships in general are consistent with worldwide observations and first principles. Globally, plants tend to have higher LMA values associated with higher temperatures and lower amounts of rainfall (Wright et al. 2005), consistent with my findings in *Protea*. High values of LMA indicate that leaves are thick, tough, and in general fairly small and resource conservative (Wright et al. 2004), constructed to withstand high and dry environments (Parkhurst and Loucks 1972; Moles et al. 2014). Additionally, many traits appear to exhibit similar adaptations to low water and low nutrient availability, resulting in overall resource conservative phenotypes (Cunningham et al. 1999; Fonseca et al. 2000). There are numerous mechanisms for achieving these traits (sclerophylly and reduced leaf size) (McDonald et al. 2003; Nicotra et al. 2011), and in Proteaceae they are likely linked to open habitats (Jordan et al. 2005). Moreover, the evolution of these scleromorphic traits may have allowed Proteaceae in open habitats to diversify more rapidly than groups in closed, forested habitats (Onstein et al. 2016).

Some of my results are consistent with these expectations: *P. punctata* has higher water content with more available phosphorus (Figure 3). However, I also see lower wood density with more acidic (lower pH) soil, where more acidic soils can be the result of excess water and mineral leaching. Within angiosperms, higher wood density is generally associated with lower elevations and higher temperatures (Hacke et al. 2001; Swenson and Enquist 2007), so I would have expected the opposite: higher wood density associated with acidic soils.

DISSECTING THE CAUSES OF TRAIT-ENVIRONMENT RELATIONSHIPS

If there are genetically-linked adaptive differences across my gradient related to traits, I expect to find relationships in controlled conditions consistent with those found in the field. My inability to detect any trait-environment relationships in the greenhouse suggests that phenotypic plasticity may be responsible for the observed patterns in the field (Figure 4, Table S4). Trait-relationships at this scale have previously been attributed to intraspecific variation or changes in patterns of variation (Ackerly et al.

2002; Lajoie and Vellend 2015; Mitchell et al. 2016). Phenotypic plasticity that enhances survival or reproduction is adaptive (Schlichting 1986; Schlichting and Pigliucci 1998), and may be especially relevant for traits related to water use (Nicotra and Davidson 2010). Plasticity has been demonstrated in physiological traits associated with elevational gradients (Cordell et al. 1998), and LMA, for instance, responds plastically to water stress, temperature, nutrient availability, and light environment (Poorter et al. 2009). In this greenhouse experiment, I detected plasticity between control and dry-down treatments in seedling leaf and overall size in *P. punctata,* but not in *P. venusta* or any other traits. In the field *P. punctata* at lower elevations tended to have bigger leaves. Lower elevations associated with higher temperatures in the field could indicate increased drought stress, but I do not have enough evidence to claim that the leaf size plasticity in the experiment is necessarily adaptive.

Differences between our field and greenhouse observations might reflect trait differences related to ontogeny, if not due to environmentally-induced plasticity. Trait differences may be less apparent in seedlings than in adults (Mediavilla et al. 2013), and functional traits within an individual can vary drastically among ontogenetic stages (Mason et al. 2013). Still, differences in seedling traits of single species have been detected across genotypes within species or hybrid groups, but typically only across genotypes from drastically different environments (Abrams et al. 1990; Meng et al. 2015). For Mediterranean regions, Davis (1986) predicted that stress is highest at the seedling stage, and that interspecific differences should be greatest early on. Previous common-garden work in *Protea* has found relationships at early life history stages consistent with field correlations (Carlson et al. 2011; Prunier et al. 2012), as well as evidence for plasticity in leaf size and stomatal traits (Carlson and Holsinger 2012).

In both species, I find weak trends (not significant) that suggest that seedlings from plants found at higher elevation do better than those from plants at lower elevations (Figure 6A). This could indicate a filter related to elevation, where plants at high elevations are those that are able to grow quickly as seedlings, and could be moderated by overall high root:shoot biomass ratios (Figure 6B). However, this does not appear to be related to water stress, as the relative performance of plants in the dry-down compared to control in my greenhouse study was not related to maternal position (Figure 6A).

The consequences of trait-environment relationships are highly dependent on their underlying causes. If environmental filtering structures trait-environment associations, I would expect to see extremely localized persistence of only individuals capable of establishing in specific microhabitats. My results more strongly support a role for phenotypic plasticity than environmental filtering at this microclimatic scale. Species trait-environment relations are not apparent under experimental conditions, and induced stress does not have predictable effects on seedling survival or performance, though plasticity between treatments is limited. This plasticity may be adaptive, as some of the relationships detected in this local site are in the same direction as presumably adaptive associations across populations and species as detected using common garden experiments (Carlson et al. 2011; Prunier et al. 2012; Carlson et al. 2015). Additionally, plasticity may be important in the future, as the CFR is predicted to become slightly hotter under future climate change, but more importantly many areas along the coast are predicted to experience a significant decrease in rainfall (Affairs 2011; Altwegg et al. 2014). The ability to respond plastically to drought stress may allow species to persist in their current geographic range, but will likely be species-specific.

CAVEATS

I assume that drought intensity is limiting establishment in this system, but there are a number of environmental filters that act in this region, including rainfall seasonality, temperature, and fire regime. Here I chose to experimentally manipulate the environment with respect to water stress, but at high elevation sites such as Blesberg, low temperatures or even snow might be more important filters. Additionally, drought stress may increase with increasing elevation, though this may not be the case as average relative humidity is also higher at higher elevations (Table 2). The near impossibility of measuring root traits on adults in the field also does not allow us to fully characterize adult water strategies. Timing could also be an issue: other work has examined the effects of water stress even earlier during ontogeny and found significant (and species-specific) effects on germination rates (Mustart et al. 2012).

My inability to detect trait-environment relationships in the greenhouse could be affected by factors in my experiment. Greenhouse-grown plants overall often have different attributes from those grown outdoors (Poorter et al. 2016), while my set-up in particular also has some interesting points. Although dry-down plants showed some indication of lowering their stomatal conductance, these plants actually did better than their control counterparts. This does not seem likely due to overwatering in the control, as the water content of the pots was well within the range of previous experiments in *Protea* (Heschel et al. 2014) and healthy adult plants (pers. observation). Additionally, I only had sufficient seed per mother for 33 maternal lines, which reduced my ability to detect relationships.

CONCLUSIONS

The causes and consequences of trait-environment relationships remain elusive, and are different at different ecological, spatial, and evolutionary scales. I found evidence for these relationships at the micro-scale, within a span of a few hundred meters, in South African shrubs in a biodiversity hotspot. However, these relationships were different in closely related species, pointing to the potential for species-specific strategies. My experimental approach did not find evidence for these relationships in controlled settings, suggesting that the observed patterns may be due to phenotypic plasticity in the field, with limited evidence for environmental filtering in both species associated with higher elevations. I detected some plasticity in terms of seedling leaf and overall size in the dry-down experiment, but not in other functional traits. I conclude that intraspecific trait-environment relationships at the microscale are likely due to plasticity with a minor role of environmental filtering at the seedling stage associated with elevation, and that interspecific differences reflect a variety of strategies in response to the same climatic factors. Additional work on the genetic architecture underlying these traits and more extensive experiments to isolate the causal environmental factors is needed to verify these results.

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Author Contributions

N. Mitchell designed this study, collected field samples, carried out the greenhouse experiment, ran the analyses, and wrote this chapter. K.E. Holsinger wrote initial versions of many of the models, assisted in framing this work, and contributed substantially to the revisions.

Tables

Table 1. Traits and environmental variables used in this study.

Trait	Description
HEIGHT	plant height (cm)
CANOPY	canopy area (cm^2)
LMA	leaf mass per area ($g \cdot cm^{-2}$)
WOOD	wood density
LWR	leaf length-to-width ratio
FWC	leaf fresh water content ($g \cdot g_{dw}^{-1}$)
SD	stomatal density (stomates $\cdot cm^{-2}$)
NMASS	leaf nitrogen per mass (%)
D13C	leaf ^{13}C:^{12}C (‰)
CNRATIO	leaf carbon:nitrogen ratio
PERFORMANCE	total biomass (g)
RSratio	root biomass (g) : shoot biomass (g)
CONDUCTANCE	stomatal conductance, mmol $\cdot m^{-2} \cdot s^{-1}$
Environment	**Description**
ASPECT	east-westness (radians)
ELEV	elevation (m)
INSOL	Insolation (W/m^2)
AVGRH	mean relative humidity (%)
AVGT	mean temperature (°C)
MAXT	maximum temperature (°C)
SOILP	phosphorus in soil (mg/kg)
SOILpH	pH of soil

Table 2. Environment – location modeling results. Means, 95% credible intervals, and 80% credible intervals. Significant associations in bold.

ENVI	LOCATION	Mean	95% CI	80% CI
AVGRH	ASPECT	-0.163	[-0.754, 0.426]	[-0.537, 0.215]
	ELEV	0.357	[-0.275, 0.973]	[-0.037, 0.747]
	INSOL	-0.137	[-0.593, 0.327]	[-0.416, 0.156]
AVGT	ASPECT	0.059	[-0.387, 0.518]	[-0.224, 0.342]
	ELEV	**-0.626**	**[-1.099, -0.143]**	**[-0.927, -0.314]**
	INSOL	0.213	[-0.141, 0.549]	[-0.002, 0.433]
MAXT	ASPECT	0.004	[-0.621, 0.639]	[-0.380, 0.402]
	ELEV	-0.100	[-0.746, 0.570]	[-0.512, 0.316]
	INSOL	**0.336**	[-0.174, 0.824]	**[0.018, 0.646]**
SOILP	ASPECT	-0.072	[-0.530, 0.417]	[-0.368, 0.224]
	ELEV	-0.171	[-0.674, 0.377]	[-0.498, 0.159]
	INSOL	0.147	[-0.261, 0.544]	[-0.112, 0.394]
SOILpH	ASPECT	**-0.537**	**[-0.965, -0.088]**	**[-0.802, -0.268]**
	ELEV	**-0.873**	**[-1.329, -0.392]**	**[-1.156, -0.587]**
	INSOL	0.180	[-0.148, 0.502]	[-0.021, 0.377]

Table 3. Trait data for greenhouse seedlings for *P. punctata* and *P. venusta*. Raw trait values, standard deviations and ranges. Units correspond to units referred to in Table 1.

P. punctata N = 179

	CONTROL N = 81		DROUGHT N = 98	
	Mean ± sd	Range	Mean ± sd	Range
SURVIVORSHIP	66%		80%	
CONDUCTANCE	135.2 ± 56.4	41.7 - 341.5	120.4 ± 77.2	29.1 - 430.7
VWC	10.8 ± 2.0	7.1 - 14.9	1.2 ± 0.0	0.0 - 3.6
FWC	2.137 ± 0.594	0.857 - 3.724	2.371 ± 0.578	0.860 - 3.717
HEIGHT	3.8 ± 0.9	1.8-6.0	4.3 ± 1.2	2.1 - 6.1
LEAFAREA	1.337 ± 0.696	0.324 - 4.132	2.258 ± 1.682	0.235 -7.646
LMA	0.010 ± 0.003	0.004 - 0.021	0.010 ± 0.003	0.004 - 0.020
LWR	2.161 ± 0.292	1.568 - 3.034	2.237 ± 0.308	1.481 - 3.068
SD	59.120 ± 11.639	39.832 - 102.725	59.159 ± 12.202	35.639 - 102.725
ROOT_BIOMASS	0.087 ± 0.037	0.022 - 0.179	0.131 ± 0.081	0.008 - 0.469
STEM_BIOMASS	0.123 ± 0.068	0.021 - 0.392	0.229 ± 0.186	0.022-0.719
TOTAL_BIOMASS	0.211 ± 0.096	0.047 - 0.571	0.360 ± 0.260	0.047 - 0.571
RSRATIO	0.773 ± 0.299	0.305 - 2.022	0.720 ± 0.360	0.257 - 1.960

P. venusta N = 156

	CONTROL N = 71		DROUGHT N = 85	
	Mean ± sd	Range	Mean ± sd	Range
SURVIVORSHIP	75%		88%	
CONDUCTANCE	142.1 ± 53.8	52.7 - 294.1	95.0 ± 64.8	32.9 - 342.7
VWC	10.7 ± 1.9	6.9 - 14.4	1.2 ± 0.9	0.0 - 3.6
FWC	2.786 ± 0.722	1.197 - 4.745	2.634 ± 0.626	1.156 - 3.715
HEIGHT	3.8 ± 0.8	2.3 - 6.1	4.0 ± 0.9	2.0 - 7.1
LEAFAREA	1.270 ± 0.539	0.334 - 2.861	1.515 ± 0.854	0.250 - 4.549
LMA	0.011 ± 0.003	0.004-0.026	0.011 ± 0.004	0.006 - 0.026
LWR	2.198 ± 0.257	1.675 - 2.792	2.255 ± 0.369	1.502 - 3.740
SD	50.019 ± 9.471	31.447 - 85.954	52.186 ± 10.690	37.736 - 98.532
ROOT_BIOMASS	0.094 ± 0.033	0.027 - 0.179	0.120 ± 0.068	0.068 - 0.348
STEM_BIOMASS	0.146 ± 0.090	0.020 - 0.449	0.215 ± 0.181	0.033 - 0.944
TOTAL_BIOMASS	0.241 ± 0.116	0.073 - 0.595	0.335 ± 0.241	0.060 - 1.176
RSRATIO	0.787 ± 0.376	0.297 - 2.650	0.707 ± 0.300	0.246 - 1.679

Table 4. Survival model results: mean and credible intervals for coefficients related to maternal elevation and intercepts estimated for each treatment. Means, 95%, and 80% credible intervals. Significant associations in bold.

	P. punctata			*P. venusta*		
	Mean	95% CI	80% CI	Mean	95% CI	80% CI
elev_control	0.109	[-0.516, 0.756]	[-0.304, 0.512]	0.490	[-0.305, 1.306]	[-0.042, 1.036]
elev_dry-down	-0.201	[-0.919, 0.497]	[-0.662, 0.245]	-0.116	[-1.000, 0.787]	[-0.692, 0.467]
intercept_control	0.109	[-1.249, 1.502]	[-0.801, 1.026]	0.143	[-1.262, 1.514]	[-0.785, 1.056]
intercept_	0.772	[-0.647, 2.133]	[-0.158, 1.681]	**1.208**	[-0.232, 2.644]	**[0.255, 2.180]**

Figures

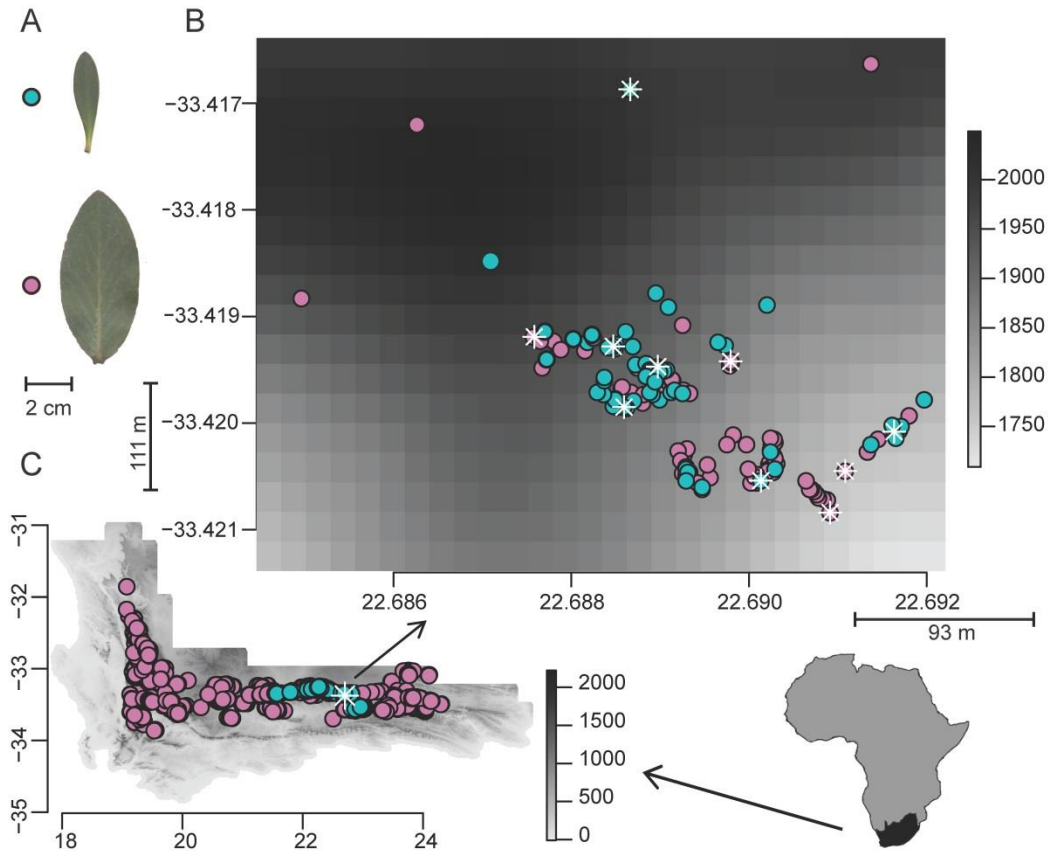

Figure 1. Examples of leaves for each species (A), sampling locations at the study site, Blesberg Mountain (B), and observations for each species from the Protea Atlas (proteaatlas.org.za) (C). *P. punctata* = pink-filled circles, *P. venusta* = blue-filled circles, microsites = white asterisk.

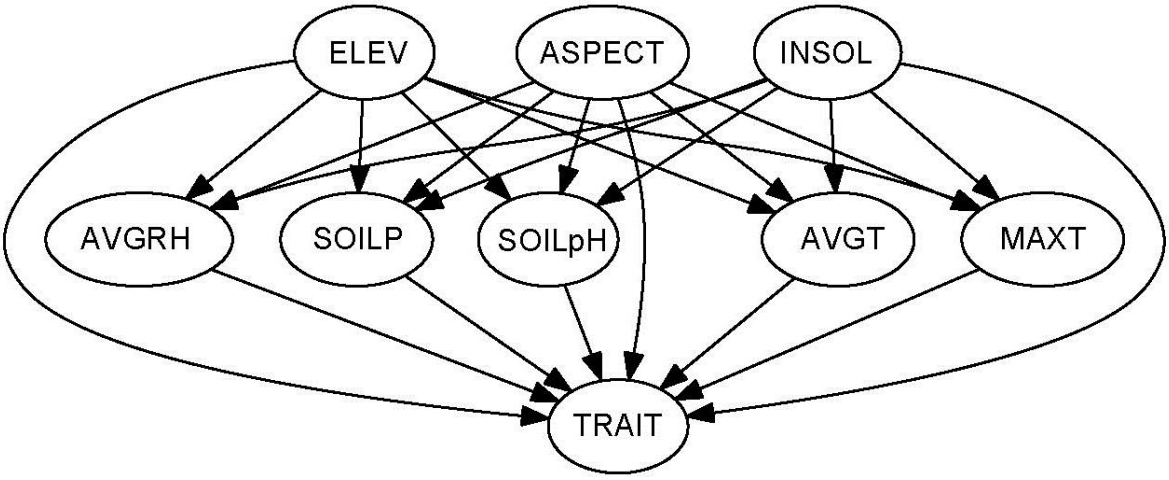

Figure 2. Diagrammatic representation of the Bayesian path model for both field and greenhouse trait-environment relationships. For ELEV, ASPECT, AND INSOL, n = 150; for AVGRH, SOILP, SOILpH, AVGT, MAXT, n = 10; for TRAIT, n = 150.

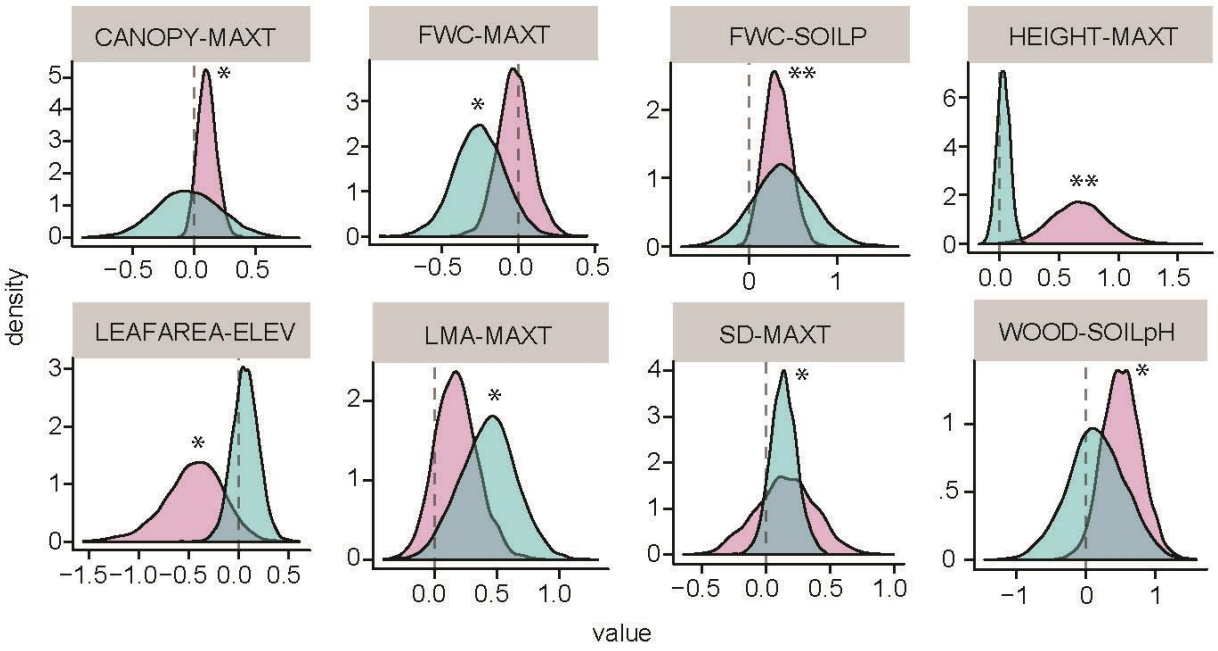

Figure 3. Density plots of posterior distributions of field-measured trait-environment relationships for which a relationship in at least one species was detected. *P. punctata* in pink, *P. venusta* in blue. Significance values: * = significant at 80% credible level, ** = significant at 95% credible level. See Table S3 for full results.

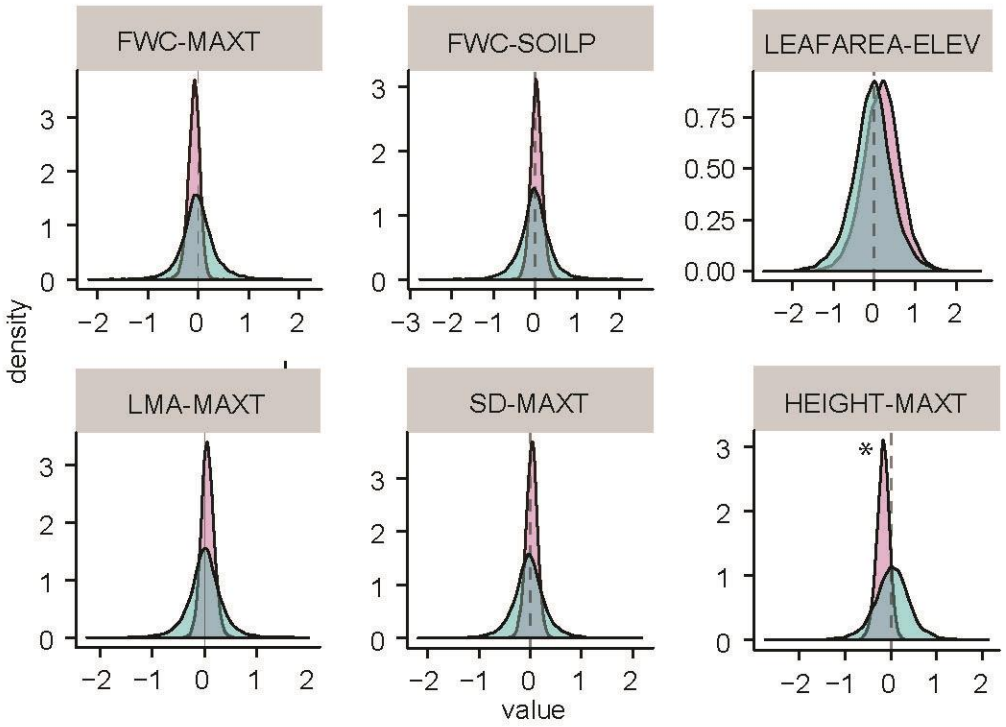

Figure 4. Density plots of posterior distributions of greenhouse-measured trait-environment relationships for which there was a significant relationship in the field. Relationships with WOOD and CANOPY are not included, as they were not measured in the greenhouse. *P. punctata* in pink, *P. venusta* in blue. Significance values: * = significant at 80% credible level. See Table S4 for full results.

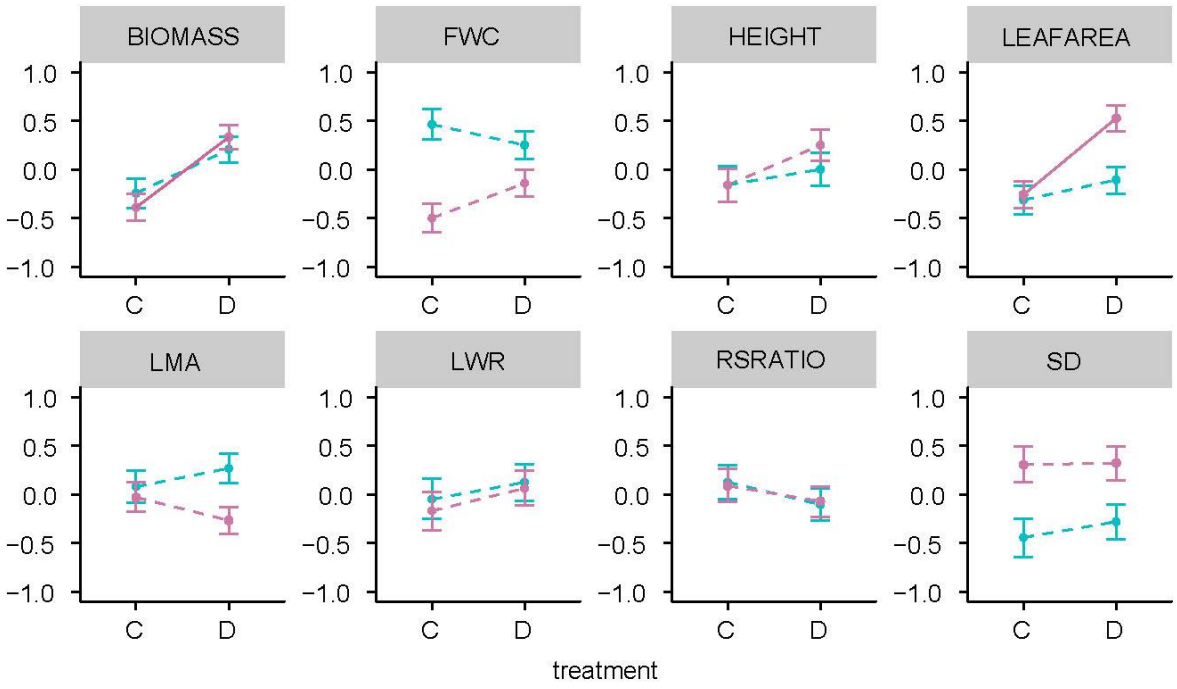

Figure 5. Reaction norms for seedling traits measured in either the control (C) or dry-down (D) treatments. Points are modeled means, bars are 95% credible intervals. Solid lines are significant at the 80% credible level, dashed lines are not significant. *P. punctata* in pink, *P. venusta* in blue.

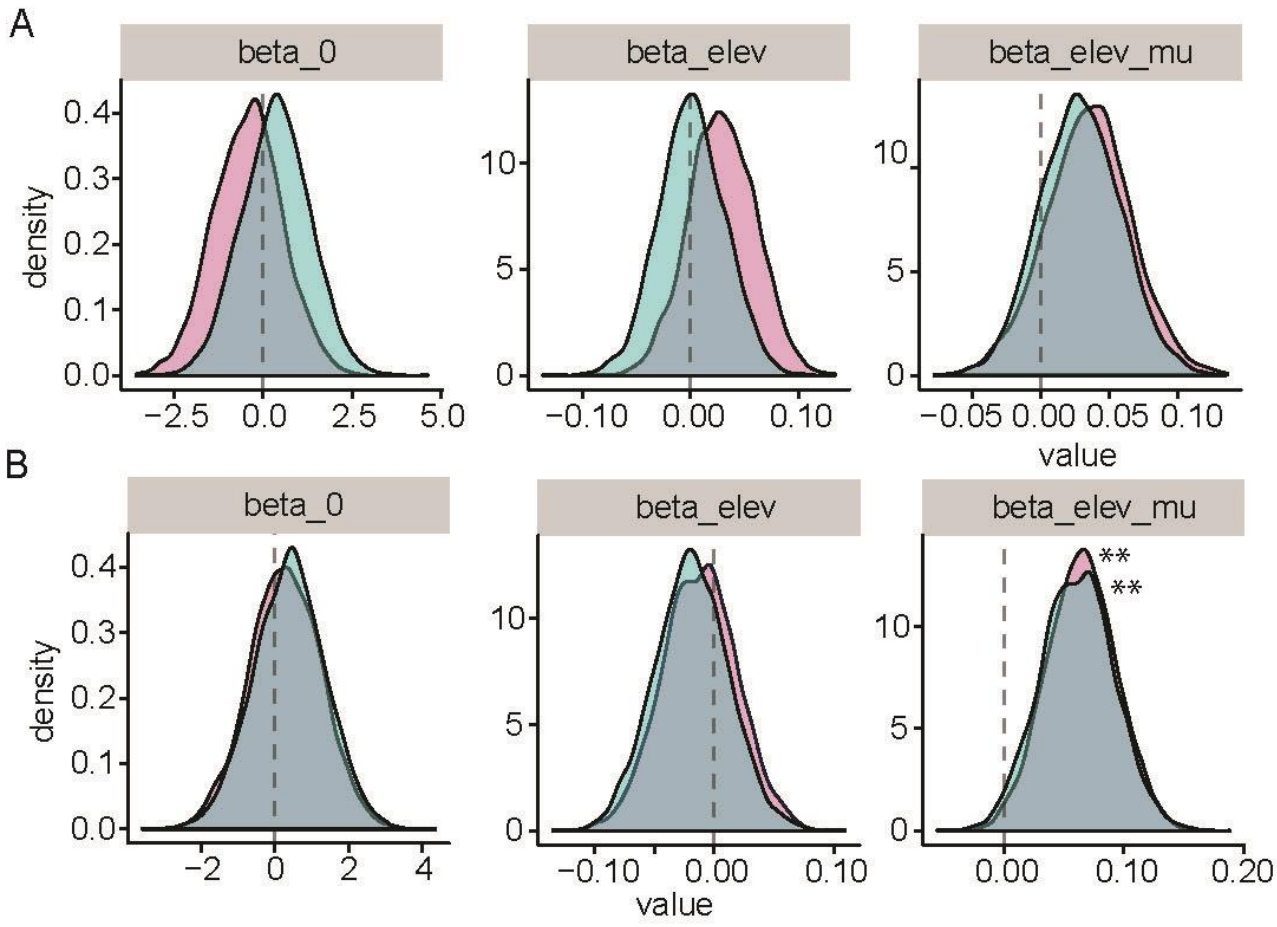

Figure 6. Density plots of posterior distributions of relationships between either performance (total biomass, A) or Root:Shoot ratio (B) and the effect of treatment on relative performance/RSratio (beta_0), the effect of elevation on relative performance/RSratio (beta_elev), and the effect of elevation on overall performance/RSratio (beta_elev_mu). *P. punctata* in pink, *P. venusta* in blue. Significance values: ** = significant at 95% credible level.

Supplemental Materials

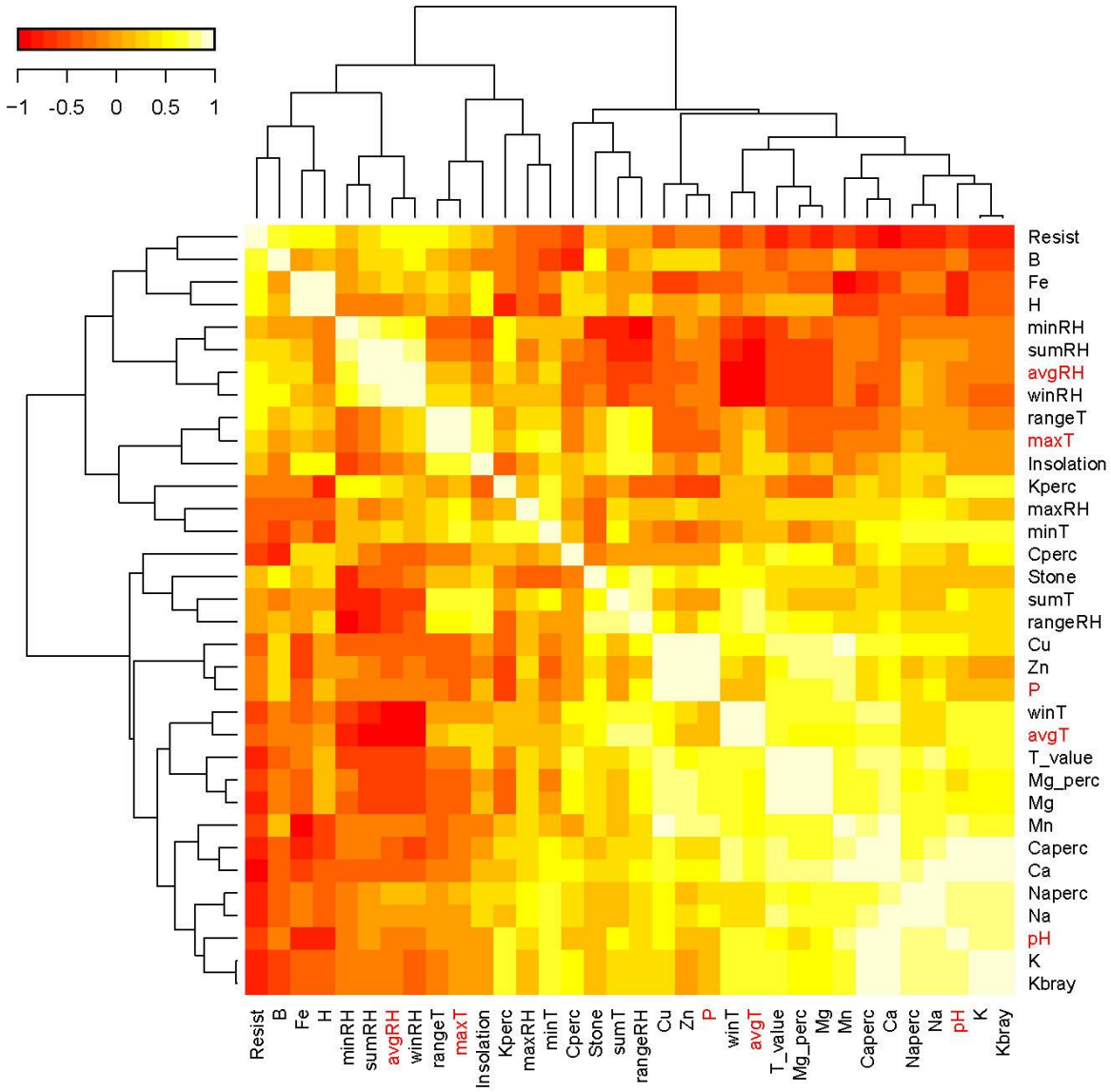

Figure S1. Heatmap of environmental correlations used for variable selection. Variables highlighted in red were used for analysis. Color indicates strength of correlation.

Table S1. Field data for environmental variables (means, standard deviations, and ranges)

Variable	Mean ± sd	Range
RH (%)	64.38 ± 6.0343	55.825 - 74.894
AVGT (°C)	9.922 ± 0.896	8.077 - 10.980
MAXT (°C)	36.094 ± 3.962	31.667 - 45.278
SOILP (mg/kg)	8.8 ± 5.095	4 - 21
SOILpH	3.42 ± 0.394	2.9 - 4.2

Table S2. Field trait and location data for all individuals, *P. punctata* and *P. venusta* (means, standard deviations, and ranges)

	P. punctata (N=86)		*P. venusta* (N=61)	
Trait	Mean ± sd	Range	Mean ± sd	Range
CANOPY (cm^2	11498.11 ± 13466.91	1570.80 - 75398.22	58868.85 ± 61580.241	345.58 - 325154.84
CNRATIO	88.61 ± 13.94	61.65 - 112.33	80.50 ± 14.04	63.38 - 104.41
D13C (‰	-27.48 ± 1.13	-29.26 - 26.27	-27.41 ± 0.79	-28.71 - -26.27
FWC (g·gdw-1	1.14 ± 0.12	0.93 - 1.81	1.49 ± 0.20	0.94 - 1.95
HEIGHT (cm	110.988 ± 37.383	45 - 300	38.869 ± 11.776	11 - 80
LA (cm^2	11.675 ± 3.908	5.027 - 21.967	3.516 ± 0.984	1.713 - 6.676
LMA ($g \cdot cm^{-2}$	0.0290 ± 0.0033	0.0225 - 0.0395	0.0277 ± 0.0048	0.0190 - 0.0382
LWR	2.23 ± 0.32	1.62 - 3.31	2.98 ± 0.44	2.04 - 4.50
NMASS (%	0.53 ± 0.11	0.27 - 0.69	0.52 ± 0.18	0.31 - 0.70
SD (stomates $\cdot cm^{-2}$	65.607 ± 11.672	41.18 - 111.083	48.481 ± 7.474	31.439 - 62.877
WOOD ($g \cdot cm^3$	0.448 ± 0.033	0.382 - 0.568	0.446 ± 0.033	0.329 - 0.512
Location	Mean ± sd	Range	Mean ± sd	Range
ASPECT (radians	2.263 ± 0.600	0.096 - 6.208	2.311 ± 0.410	0.575 - 3.475
ELEV (m	1842.7 ± 62.9	1743 - 2031	1890.8 ± 54.6	1763 - 2018
INSOL ($W \cdot m^{-2}$	882823 ± 283337	208210 - 109032	951333 ± 236727	17761 - 1093193

Table S3. Field trait-environment results, means and 95% and 80% credible intervals for the estimated regression coefficient between traits and environments in the field.

		P. punctata			*P. venusta*			Species Comp	
Trait	Envi	Mean	95% CI	80% CI	Mean	95% CI	80% CI	Same	Diff
CANOPY	ASPECT	-0.018	[-0.162, 0.128]	[-0.110, 0.074]	-0.167	[-0.896, 0.553]	[-0.649, 0.306]	0.540	0.460
CANOPY	AVGRH	0.020	[-0.112, 0.160]	[-0.066, 0.111]	-0.019	[-0.861, 0.832]	[-0.582, 0.557]	0.513	0.487
CANOPY	AVGT	-0.006	[-0.201, 0.190]	[-0.128, 0.115]	-0.071	[-1.221, 1.044]	[-0.828, 0.676]	0.509	0.491
CANOPY	ELEV	0.073	[-0.173, 0.336]	[-0.090, 0.239]	-0.067	[-1.308, 1.102]	[-0.863, 0.732]	0.512	0.489
CANOPY	INSOL	-0.041	[-0.197, 0.105]	[-0.141, 0.053]	0.070	[-0.552, 0.694]	[-0.338, 0.477]	0.475	0.525
CANOPY	MAXT	0.108	[-0.034, 0.267]	**[0.014, 0.208]**	-0.046	[-0.561, 0.476]	[-0.382, 0.298]	0.443	0.557
CANOPY	SOILP	0.006	[-0.123, 0.143]	[-0.075, 0.089]	0.082	[-0.880, 1.051]	[-0.546, 0.712]	0.514	0.487
CANOPY	SOILPH	-0.042	[-0.214, 0.133]	[-0.156, 0.072]	-0.018	[-1.095, 1.019]	[-0.723, 0.661]	0.531	0.469
CNRATIO	ASPECT	-0.326	[-1.424, 0.761]	[-1.002, 0.368]	-0.164	[-1.797, 1.463]	[-1.247, 0.920]	0.561	0.440
CNRATIO	AVGRH	-0.321	[-1.313, 0.640]	[-0.938, 0.315]	-0.479	[-2.186, 1.322]	[-1.592, 0.646]	0.655	0.345
CNRATIO	AVGT	-0.039	[-1.383, 1.268]	[-0.905, 0.817]	0.147	[-1.838, 2.097]	[-1.152, 1.429]	0.599	0.402
CNRATIO	ELEV	-0.407	[-1.967, 1.218]	[-1.430, 0.618]	-0.243	[-2.202, 1.788]	[-1.526, 1.065]	0.629	0.372
CNRATIO	INSOL	0.308	[-0.923, 1.444]	[-0.457, 1.056]	-0.220	[-1.798, 1.391]	[-1.240, 0.801]	0.491	0.509
CNRATIO	MAXT	0.115	[-0.682, 0.863]	[-0.381, 0.579]	0.025	[-1.741, 1.836]	[-1.125, 1.165]	0.545	0.455
CNRATIO	SOILP	-0.020	[-0.733, 0.707]	[-0.483, 0.423]	-0.106	[-1.983, 1.798]	[-1.296, 1.080]	0.555	0.445
CNRATIO	SOILPH	-0.594	[-1.668, 0.508]	[-1.288, 0.102]	-0.549	[-2.210, 1.127]	[-1.609, 0.528]	0.720	0.280
D13C	ASPECT	-0.536	[-1.705, 0.661]	[-1.284, 0.247]	-0.204	[-1.817, 1.424]	[-1.258, 0.847]	0.583	0.417
D13C	AVGRH	-0.008	[-1.140, 1.094]	[-0.745, 0.690]	0.050	[-1.758, 1.704]	[-1.050, 1.098]	0.557	0.444
D13C	AVGT	-0.129	[-1.606, 1.424]	[-1.093, 0.829]	-0.063	[-1.946, 1.907]	[-1.284, 1.173]	0.585	0.415
D13C	ELEV	0.239	[-1.563, 1.962]	[-0.894, 1.333]	0.114	[-1.891, 2.136]	[-1.167, 1.416]	0.620	0.380
D13C	INSOL	-0.149	[-1.417, 1.128]	[-0.960, 0.678]	-0.039	[-1.580, 1.479]	[-1.061, 0.963]	0.530	0.470
D13C	MAXT	-0.239	[-1.117, 0.660]	[-0.800, 0.341]	-0.034	[-1.699, 1.679]	[-1.099, 1.038]	0.551	0.449
D13C	SOILP	-0.186	[-0.999, 0.644]	[-0.710, 0.351]	-0.056	[-1.801, 1.673]	[-1.146, 1.043]	0.552	0.448
D13C	SOILPH	0.029	[-1.177, 1.191]	[-0.750, 0.788]	0.061	[-1.600, 1.646]	[-0.973, 1.097]	0.593	0.407
FWC	ASPECT	-0.017	[-0.286, 0.225]	[-0.188, 0.140]	0.044	[-0.414, 0.501]	[-0.251, 0.344]	0.549	0.452
FWC	AVGRH	-0.074	[-0.327, 0.153]	[-0.227, 0.072]	0.075	[-0.435, 0.615]	[-0.264, 0.424]	0.532	0.469

FWC	AVGT	-0.032	[-0.364, 0.290]	[-0.242, 0.177]	-0.314	[-1.074, 0.400]	[-0.803, 0.160]	0.615	0.386
FWC	ELEV	-0.137	[-0.593, 0.297]	[-0.425, 0.136]	-0.202	[-1.008, 0.626]	[-0.741, 0.346]	0.647	0.353
FWC	INSOL	-0.098	[-0.356, 0.148]	[-0.257, 0.058]	-0.149	[-0.564, 0.267]	[-0.418, 0.122]	0.689	0.312
FWC	MAXT	-0.022	[-0.235, 0.202]	[-0.161, 0.116]	**-0.254**	[-0.574, 0.064]	**[-0.456, -0.047]**	0.590	0.410
FWC	SOILP	**0.320**	**[0.038, 0.636]**	**[0.128, 0.517]**	0.384	[-0.295, 1.083]	[-0.053, 0.826]	**0.868**	0.132
FWC	SOILPH	-0.116	[-0.422, 0.170]	[-0.306, 0.071]	-0.101	[-0.755, 0.579]	[-0.545, 0.331]	0.613	0.387
HEIGHT	ASPECT	-0.034	[-0.449, 0.390]	[-0.303, 0.228]	0.035	[-0.136, 0.210]	[-0.075, 0.143]	0.490	0.511
HEIGHT	AVGRH	0.059	[-0.334, 0.452]	[-0.194, 0.314]	0.002	[-0.146, 0.155]	[-0.094, 0.099]	0.498	0.502
HEIGHT	AVGT	-0.010	[-0.538, 0.511]	[-0.353, 0.320]	0.012	[-0.202, 0.236]	[-0.117, 0.152]	0.503	0.497
HEIGHT	ELEV	0.028	[-0.682, 0.741]	[-0.438, 0.488]	0.094	[-0.196, 0.374]	[-0.087, 0.272]	0.529	0.471
HEIGHT	INSOL	-0.218	[-0.653, 0.202]	[-0.498, 0.064]	-0.070	[-0.216, 0.075]	[-0.163, 0.022]	0.725	0.275
HEIGHT	MAXT	**0.670**	**[0.223, 1.145]**	**[0.375, 0.967]**	0.030	[-0.077, 0.139]	[-0.040, 0.100]	0.707	0.293
HEIGHT	SOILP	-0.038	[-0.405, 0.316]	[-0.265, 0.183]	-0.010	[-0.175, 0.151]	[-0.109, 0.090]	0.501	0.499
HEIGHT	SOILPH	-0.091	[-0.558, 0.397]	[-0.390, 0.219]	-0.004	[-0.231, 0.219]	[-0.145, 0.136]	0.495	0.506
LEAFAREA	ASPECT	-0.116	[-0.496, 0.184]	[-0.336, 0.083]	0.007	[-0.149, 0.157]	[-0.091, 0.102]	0.485	0.515
LEAFAREA	AVGRH	0.063	[-0.230, 0.364]	[-0.124, 0.255]	-0.016	[-0.161, 0.122]	[-0.107, 0.072]	0.483	0.517
LEAFAREA	AVGT	-0.071	[-0.501, 0.346]	[-0.351, 0.199]	0.043	[-0.149, 0.250]	[-0.083, 0.166]	0.466	0.535
LEAFAREA	ELEV	**-0.451**	[-1.100, 0.082]	**[-0.842, -0.093]**	0.068	[-0.190, 0.330]	[-0.098, 0.236]	0.324	0.676
LEAFAREA	INSOL	0.055	[-0.247, 0.378]	[-0.140, 0.255]	-0.006	[-0.139, 0.128]	[-0.091, 0.079]	0.494	0.507
LEAFAREA	MAXT	-0.029	[-0.322, 0.258]	[-0.211, 0.148]	0.008	[-0.083, 0.099]	[-0.050, 0.068]	0.508	0.492
LEAFAREA	SOILP	-0.092	[-0.414, 0.188]	[-0.293, 0.097]	-0.005	[-0.155, 0.144]	[-0.099, 0.087]	0.527	0.473
LEAFAREA	SOILPH	0.039	[-0.375, 0.405]	[-0.216, 0.276]	0.018	[-0.182, 0.227]	[-0.110, 0.147]	0.536	0.464
LMA	ASPECT	-0.036	[-0.404, 0.314]	[-0.271, 0.186]	-0.039	[-0.687, 0.605]	[-0.437, 0.364]	0.558	0.442
LMA	AVGRH	0.066	[-0.273, 0.431]	[-0.161, 0.291]	-0.096	[-0.831, 0.649]	[-0.581, 0.395]	0.527	0.473
LMA	AVGT	-0.052	[-0.563, 0.436]	[-0.371, 0.258]	0.342	[-0.634, 1.335]	[-0.306, 0.986]	0.503	0.497
LMA	ELEV	-0.132	[-0.770, 0.483]	[-0.533, 0.248]	0.248	[-0.826, 1.342]	[-0.462, 0.978]	0.506	0.495
LMA	INSOL	0.127	[-0.239, 0.473]	[-0.097, 0.349]	0.020	[-0.51, 0.553]	[-0.330, 0.376]	0.561	0.440
LMA	MAXT	0.177	[-0.141, 0.537]	[-0.035, 0.396]	**0.432**	[-0.008, 0.867]	**[0.138, 0.717]**	**0.840**	0.161
LMA	SOILP	-0.119	[-0.468, 0.199]	[-0.339, 0.093]	-0.188	[-1.068, 0.674]	[-0.762, 0.394]	0.629	0.371
LMA	SOILPH	-0.223	[-0.670, 0.204]	[-0.512, 0.050]	0.004	[-0.901, 0.964]	[-0.606, 0.612]	0.525	0.475
LWR	ASPECT	-0.125	[-0.449, 0.158]	[-0.327, 0.060]	-0.138	[-0.653, 0.373]	[-0.472, 0.195]	0.653	0.347

LWR	AVGRH	-0.019	[-0.324, 0.284]	[-0.213, 0.178]	0.060	[-0.471, 0.604]	[-0.301, 0.415]	0.631	0.369
LWR	AVGT	-0.006	[-0.441, 0.421]	[-0.285, 0.271]	-0.050	[-0.800, 0.698]	[-0.541, 0.435]	0.642	0.358
LWR	ELEV	-0.135	[-0.685, 0.373]	[-0.486, 0.209]	-0.054	[-0.904, 0.822]	[-0.607, 0.507]	0.613	0.387
LWR	INSOL	0.080	[-0.203, 0.364]	[-0.101, 0.260]	0.033	[-0.389, 0.463]	[-0.242, 0.310]	0.588	0.412
LWR	MAXT	0.066	[-0.198, 0.350]	[-0.113, 0.246]	-0.017	[-0.354, 0.301]	[-0.231, 0.192]	0.563	0.438
LWR	SOILP	0.071	[-0.217, 0.354]	[-0.115, 0.254]	0.122	[-0.488, 0.707]	[-0.272, 0.505]	0.686	0.314
LWR	SOILPH	-0.122	[-0.505, 0.244]	[-0.361, 0.123]	-0.050	[-0.766, 0.649]	[-0.518, 0.427]	0.617	0.384
NMASS	ASPECT	0.335	[-0.695, 1.275]	[-0.304, 0.942]	0.418	[-1.178, 2.059]	[-0.651, 1.506]	0.629	0.371
NMASS	AVGRH	0.171	[-0.614, 0.995]	[-0.338, 0.682]	-0.065	[-1.754, 1.759]	[-1.123, 1.026]	0.522	0.479
NMASS	AVGT	0.082	[-0.956, 1.180]	[-0.599, 0.777]	-0.062	[-2.002, 1.887]	[-1.314, 1.158]	0.547	0.454
NMASS	ELEV	-0.160	[-1.595, 1.281]	[-1.066, 0.781]	0.420	[-1.592, 2.420]	[-0.896, 1.716]	0.539	0.461
NMASS	INSOL	0.132	[-0.906, 1.211]	[-0.549, 0.815]	0.494	[-1.079, 2.063]	[-0.512, 1.493]	0.570	0.430
NMASS	MAXT	0.171	[-0.481, 0.831]	[-0.233, 0.583]	-0.116	[-1.856, 1.626]	[-1.201, 0.993]	0.493	0.507
NMASS	SOILP	0.192	[-0.410, 0.825]	[-0.200, 0.584]	0.041	[-1.714, 1.850]	[-1.055, 1.158]	0.516	0.484
NMASS	SOILPH	0.419	[-0.532, 1.358]	[-0.185, 1.011]	-0.420	[-2.021, 1.180]	[-1.446, 0.633]	0.415	0.585
SD	ASPECT	0.024	[-0.418, 0.456]	[-0.261, 0.299]	-0.058	[-0.404, 0.281]	[-0.280, 0.158]	0.506	0.494
SD	AVGRH	0.027	[-0.476, 0.528]	[-0.313, 0.369]	0.102	[-0.234, 0.431]	[-0.116, 0.316]	0.506	0.494
SD	AVGT	0.121	[-0.633, 0.804]	[-0.357, 0.577]	-0.104	[-0.583, 0.352]	[-0.393, 0.193]	0.448	0.552
SD	ELEV	0.318	[-0.460, 1.125]	[-0.198, 0.834]	-0.241	[-0.858, 0.331]	[-0.618, 0.121]	0.340	0.660
SD	INSOL	0.072	[-0.359, 0.512]	[-0.207, 0.347]	-0.027	[-0.294, 0.248]	[-0.206, 0.153]	0.490	0.510
SD	MAXT	0.150	[-0.312, 0.602]	[-0.158, 0.449]	**0.136**	[-0.068, 0.352]	**[0.005, 0.269]**	0.701	0.300
SD	SOILP	0.028	[-0.432, 0.459]	[-0.268, 0.319]	-0.063	[-0.446, 0.304]	[-0.288, 0.171]	0.486	0.514
SD	SOILPH	0.156	[-0.412, 0.727]	[-0.219, 0.536]	0.018	[-0.443, 0.496]	[-0.278, 0.315]	0.520	0.480
WOOD	ASPECT	0.084	[-0.336, 0.545]	[-0.203, 0.383]	0.006	[-0.544, 0.551]	[-0.356, 0.356]	0.537	0.464
WOOD	AVGRH	0.030	[-0.390, 0.452]	[-0.235, 0.299]	-0.015	[-0.594, 0.576]	[-0.400, 0.367]	0.531	0.469
WOOD	AVGT	0.144	[-0.436, 0.748]	[-0.225, 0.523]	0.287	[-0.538, 1.115]	[-0.251, 0.822]	0.605	0.395
WOOD	ELEV	0.471	[-0.245, 1.296]	[-0.014, 0.973]	0.078	[-0.918, 1.025]	[-0.536, 0.680]	0.575	0.425
WOOD	INSOL	0.034	[-0.368, 0.450]	[-0.230, 0.301]	-0.088	[-0.549, 0.389]	[-0.387, 0.220]	0.504	0.497
WOOD	MAXT	-0.107	[-0.523, 0.277]	[-0.378, 0.154]	0.176	[-0.202, 0.538]	[-0.063, 0.415]	0.372	0.628
WOOD	SOILP	-0.061	[-0.466, 0.331]	[-0.310, 0.181]	-0.077	[-0.744, 0.560]	[-0.504, 0.346]	0.535	0.465
WOOD	SOILPH	**0.521**	[-0.008, 1.068]	**[0.179, 0.875]**	0.156	[-0.682, 0.964]	[-0.378, 0.693]	0.643	0.357

Table S4. Greenhouse trait-environment results, means and 95% and 80% credible intervals for the estimated regression coefficient between seedling traits in the greenhouse and maternal environments in the field. For each species, "same" and "diff" refer to posterior probability that the associations match between the field and greenhouse. For the "Species Comp", they refer to the posterior probability that the greenhouse trait-environment relationships are the same or different between species.

		P. punctata					*P. venusta*					Species Comp	
Trait	Envi	Mean	95% CI	80% CI	Same	Diff	Mean	95% CI	80% CI	Same	Diff	Same	Different
FWC	ASPECT	-0.324	[-1.078, 0.352]	[-0.801, 0.130]	0.528	0.472	-0.164	[-0.878, 0.631]	[-0.605, 0.290]	0.471	0.530	0.634	0.366
FWC	AVGRH	0.087	[-0.282, 0.482]	[-0.151, 0.331]	0.413	0.587	-0.001	[-0.768, 0.770]	[-0.426, 0.424]	0.497	0.503	0.551	0.449
FWC	AVGT	-0.058	[-0.712, 0.572]	[-0.448, 0.326]	0.520	0.480	0.013	[-1.065, 1.103]	[-0.621, 0.648]	0.481	0.519	0.580	0.420
FWC	ELEV	0.110	[-0.708, 0.921]	[-0.401, 0.617]	0.456	0.545	0.227	[-0.843, 1.308]	[-0.409, 0.870]	0.445	0.555	0.607	0.393
FWC	INSOL	-0.125	[-0.622, 0.376]	[-0.441, 0.194]	0.607	0.393	0.264	[-0.398, 0.862]	[-0.113, 0.638]	0.328	0.672	0.383	0.617
FWC	MAXT	-0.088	[-0.335, 0.123]	[-0.236, 0.051]	0.552	0.448	-0.014	[-0.681, 0.714]	[-0.390, 0.373]	0.542	0.458	0.559	0.441
FWC	SOILP	0.024	[-0.244, 0.304]	[-0.146, 0.198]	0.573	0.427	-0.019	[-0.805, 0.753]	[-0.455, 0.403]	0.480	0.520	0.548	0.452
FWC	SOILpH	0.272	[-0.274, 0.864]	[-0.080, 0.637]	0.312	0.688	0.102	[-0.835, 1.078]	[-0.457, 0.686]	0.480	0.520	0.608	0.392
HEIGHT	ASPECT	0.063	[-0.795, 0.873]	[-0.477, 0.593]	0.486	0.515	0.111	[-0.678, 0.937]	[-0.379, 0.604]	0.528	0.472	0.525	0.475
HEIGHT	AVGRH	0.043	[-0.412, 0.510]	[-0.251, 0.329]	0.507	0.493	0.106	[-0.790, 0.968]	[-0.401, 0.618]	0.509	0.491	0.509	0.491
HEIGHT	AVGT	-0.038	[-0.779, 0.673]	[-0.490, 0.404]	0.518	0.482	-0.071	[-1.298, 1.163]	[-0.829, 0.685]	0.507	0.493	0.518	0.482
HEIGHT	ELEV	0.168	[-0.769, 1.123]	[-0.432, 0.755]	0.506	0.494	-0.013	[-1.166, 1.246]	[-0.744, 0.730]	0.500	0.500	0.525	0.475
HEIGHT	INSOL	0.178	[-0.388, 0.768]	[-0.185, 0.552]	0.327	0.673	-0.041	[-0.733, 0.613]	[-0.450, 0.366]	0.548	0.453	0.480	0.520
HEIGHT	MAXT	-0.173	[-0.460, 0.088]	[-0.348, -0.003]	0.097	0.904	0.022	[-0.823, 0.840]	[-0.463, 0.489]	0.525	0.475	0.472	0.528
HEIGHT	SOILP	0.067	[-0.251, 0.401]	[-0.141, 0.277]	0.482	0.518	0.052	[-0.828, 1.019]	[-0.470, 0.583]	0.499	0.502	0.508	0.592
HEIGHT	SOILpH	0.236	[-0.402, 0.902]	[-0.175, 0.656]	0.406	0.594	0.120	[-0.956, 1.197]	[-0.554, 0.792]	0.499	0.501	0.559	0.440
LEAFAREA	ASPECT	-0.345	[-1.193, 0.503]	[-0.891, 0.194]	0.658	0.342	-0.098	[-0.772, 0.580]	[-0.496, 0.309]	0.493	0.508	0.581	0.419
LEAFAREA	AVGRH	0.056	[-0.368, 0.481]	[-0.204, 0.320]	0.529	0.471	0.087	[-0.625, 0.775]	[-0.291, 0.471]	0.492	0.508	0.545	0.455
LEAFAREA	AVGT	-0.042	[-0.737, 0.625]	[-0.461, 0.369]	0.506	0.494	-0.070	[-1.103, 0.946]	[-0.675, 0.526]	0.482	0.518	0.535	0.465
LEAFAREA	ELEV	0.180	[-0.728, 1.062]	[-0.383, 0.743]	0.358	0.643	-0.052	[-1.114, 0.947]	[-0.668, 0.544]	0.483	0.517	0.519	0.481
LEAFAREA	INSOL	0.005	[-0.573, 0.605]	[-0.365, 0.381]	0.506	0.494	0.188	[-0.365, 0.730]	[-0.130, 0.511]	0.489	0.511	0.512	0.488
LEAFAREA	MAXT	-0.046	[-0.293, 0.207]	[-0.201, 0.112]	0.516	0.484	0.002	[-0.645, 0.643]	[-0.356, 0.351]	0.495	0.505	0.509	0.492
LEAFAREA	SOILP	0.032	[-0.286, 0.351]	[-0.168, 0.236]	0.456	0.545	0.007	[-0.718, 0.714]	[-0.379, 0.402]	0.493	0.507	0.500	0.501
LEAFAREA	SOILpH	0.098	[-0.514, 0.710]	[-0.288, 0.481]	0.533	0.467	0.103	[-0.843, 1.030]	[-0.422, 0.640]	0.522	0.479	0.554	0.446
LMA	ASPECT	0.420	[-0.291, 1.180]	[-0.048, 0.901]	0.442	0.558	-0.048	[-0.855, 0.675]	[-0.505, 0.398]	0.514	0.487	0.473	0.527

LMA	AVGRH	-0.039	[-0.461, 0.371]	[-0.296, 0.221]	0.483	0.518	-0.048	[-0.839, 0.664]	[-0.462, 0.368]	0.496	0.504	0.545	0.455
LMA	AVGT	-0.007	[-0.679, 0.638]	[-0.422, 0.410]	0.502	0.499	0.008	[-1.085, 1.079]	[-0.624, 0.642]	0.497	0.503	0.550	0.450
LMA	ELEV	-0.124	[-0.969, 0.763]	[-0.653, 0.426]	0.537	0.463	-0.068	[-1.145, 0.961]	[-0.706, 0.561]	0.482	0.518	0.571	0.430
LMA	INSOL	0.210	[-0.321, 0.731]	[-0.121, 0.536]	0.673	0.327	-0.107	[-0.730, 0.534]	[-0.481, 0.269]	0.485	0.515	0.420	0.580
LMA	MAXT	0.062	[-0.171, 0.312]	[-0.088, 0.218]	0.626	0.374	0.012	[-0.693, 0.705]	[-0.374, 0.391]	0.509	0.492	0.525	0.475
LMA	SOILP	0.028	[-0.267, 0.316]	[-0.156, 0.214]	0.460	0.540	0.001	[-0.815, 0.810]	[-0.436, 0.439]	0.501	0.499	0.522	0.478
LMA	SOILpH	-0.199	[-0.810, 0.397]	[-0.576, 0.185]	0.683	0.317	-0.133	[-1.118, 0.773]	[-0.707, 0.421]	0.495	0.505	0.602	0.398
LWR	ASPECT	-0.442	[-1.241, 0.325]	[-0.957, 0.060]	0.713	0.287	-0.112	[-0.979, 0.749]	[-0.643, 0.416]	0.555	0.445	0.588	0.412
LWR	AVGRH	0.090	[-0.351, 0.545]	[-0.187, 0.373]	0.498	0.502	-0.096	[-0.995, 0.819]	[-0.638, 0.454]	0.490	0.510	0.506	0.494
LWR	AVGT	-0.168	[-0.887, 0.532]	[-0.617, 0.277]	0.508	0.492	0.045	[-1.223, 1.332]	[-0.767, 0.846]	0.512	0.488	0.557	0.444
LWR	ELEV	0.242	[-0.703, 1.190]	[-0.345, 0.843]	0.424	0.577	0.047	[-1.164, 1.303]	[-0.711, 0.812]	0.487	0.513	0.573	0.428
LWR	INSOL	-0.167	[-0.733, 0.402]	[-0.526, 0.191]	0.404	0.597	0.295	[-0.392, 0.980]	[-0.136, 0.720]	0.523	0.477	0.363	0.637
LWR	MAXT	-0.063	[-0.327, 0.185]	[-0.230, 0.099]	0.439	0.562	-0.042	[-0.848, 0.797]	[-0.526, 0.447]	0.490	0.510	0.549	0.451
LWR	SOILP	0.190	[-0.118, 0.522]	[-0.011, 0.394]	0.647	0.354	-0.001	[-0.955, 0.985]	[-0.571, 0.574]	0.493	0.507	0.514	0.486
LWR	SOILpH	0.141	[-0.482, 0.793]	[-0.251, 0.547]	0.429	0.572	0.021	[-1.067, 1.175]	[-0.669, 0.722]	0.498	0.502	0.562	0.438
SD	ASPECT	0.475	[-0.278, 1.223]	[-0.005, 0.955]	0.534	0.466	-0.158	[-0.850, 0.654]	[-0.597, 0.302]	0.544	0.457	0.340	0.661
SD	AVGRH	-0.060	[-0.45, 0.3380]	[-0.309, 0.187]	0.481	0.519	-0.047	[-0.772, 0.769]	[-0.453, 0.384]	0.447	0.553	0.568	0.432
SD	AVGT	0.039	[-0.584, 0.668]	[-0.346, 0.426]	0.506	0.494	0.013	[-1.163, 1.068]	[-0.646, 0.645]	0.496	0.505	0.569	0.431
SD	ELEV	-0.282	[-1.104, 0.591]	[-0.811, 0.253]	0.355	0.646	0.026	[-0.990, 1.121]	[-0.588, 0.673]	0.492	0.509	0.548	0.452
SD	INSOL	0.220	[-0.322, 0.755]	[-0.124, 0.565]	0.583	0.417	0.122	[-0.497, 0.724]	[-0.239, 0.486]	0.477	0.523	0.617	0.383
SD	MAXT	0.027	[-0.208, 0.247]	[-0.116, 0.168]	0.553	0.447	-0.028	[-0.731, 0.661]	[-0.412, 0.353]	0.463	0.538	0.531	0.470
SD	SOILP	-0.001	[-0.285, 0.287]	[-0.186, 0.183]	0.503	0.497	-0.005	[-0.764, 0.802]	[-0.425, 0.423]	0.507	0.493	0.537	0.463
SD	SOILpH	-0.076	[-0.647, 0.516]	[-0.441, 0.285]	0.466	0.535	-0.013	[-0.952, 0.948]	[-0.580, 0.557]	0.495	0.505	0.567	0.434

Table S5. R^2 for models.

Field Trait - Envi

Trait	*P. punctata*	*P. venusta*	Total
CANOPY	0.141	0.086	0.309
CNRATIO	0.132	-0.065	0.096
D13C	0.003	-0.496	-0.107
FWC	0.241	0.385	0.698
HEIGHT	0.287	-0.041	0.699
LEAFAREA	0.178	-0.108	0.695
LMA	0.143	0.305	0.253
LWR	0.061	0.056	0.487
NMASS	0.169	0.515	0.273
SD	0.201	0.129	0.518
WOOD	0.165	0.073	0.122

Greenhouse Trait - Envi

Trait	*P. punctata*	*P. venusta*	Total
FWC	0.051	-0.02	0.116
HEIGHT	0.066	0.085	0.075
LEAFAREA	0.067	0.067	0.098
LMA	0.043	-0.026	0.032
LWR	0.063	0.078	0.069
SD	0.006	0.013	0.118

Greenhouse Treatment Plasticity

Trait	*P. punctata*	*P. venusta*	Total
FWC	0.566	0.450	0.556
HEIGHT	0.374	0.319	0.355
LEAFAREA	0.642	0.332	0.595
LMA	0.571	0.434	0.412
LWR	0.151	0.228	0.189
PERFORMANCE	0.622	0.606	0.614
RSRATIO	0.404	0.435	0.417
SD	0.196	0.094	0.250

Other

Model	*P. punctata*	*P. venusta*	Total
Survival	0.389	0.318	0.367
Performance	0.624	0.605	0.615
RSratio	0.449	0.376	0.416

Table S6. Seedling plasticity results, means and 95% and 80% credible intervals for the estimated slope of the reaction norm between control and dry-down treatments. Significant slopes are indicated in bold. For the "Species Comparison", "Same" and "Diff" refer to the posterior probability that the reaction norms are in the same or different direction between species.

	P. punctata			*P. venusta*			Species Comparison	
Trait	Mean	95% CI	80% CI	Mean	95% CI	80% CI	Same	Different
FWC	0.166	[-0.331, 0.664]	[-0.155, 0.489]	-0.166	[-0.661, 0.330]	[-0.488, 0.167]	0.507	0.493
HEIGHT	0.250	[-0.260, 0.667]	[-0.091, 0.519]	0.097	[-0.376, 0.547]	[-0.212, 0.396]	0.768	0.232
LEAFAREA	**0.459**	[-0.026, 0.946]	**[0.147, 0.783]**	0.048	[-0.445, 0.539]	[-0.269, 0.365]	0.607	0.393
LMA	-0.150	[-0.661, 0.346]	[-0.479, 0.178]	0.117	[-0.396, 0.621]	[-0.222, 0.449]	0.598	0.402
LWR	0.149	[-0.300, 0.605]	[-0.139, 0.436]	0.164	[-0.287, 0.603]	[-0.125, 0.460]	0.762	0.238
PERFORMANCE	**0.337**	[-0.159, 0.826]	**[0.014, 0.660]**	0.223	[-0.272, 0.723]	[-0.095, 0.545]	**0.876**	0.124
RSRATIO	-0.021	[-0.509, 0.478]	[-0.344, 0.305]	-0.136	[-0.632, 0.363]	[-0.456, 0.193]	0.749	0.251
SD	0.058	[-0.375, 0.489]	[-0.221, 0.339]	0.177	[-0.258, 0.613]	[-0.100, 0.453]	0.704	0.296

Table S7. Greenhouse survival, performance, and R:S ratio modeling results related to maternal elevation (means and 95% and 80% credible intervals). beta_0: species/treatment intercept, beta_elev: relative performance associated with elevation, beta_elev_mu: absolute performance associated with elevation, sd_individual: variation among individuals, sd_mother: variation within maternal lines, sd_bin: variation within bins.

		P. punctata			*P. venusta*			Same	Different
		Mean	95% CI	80% CI	Mean	95% CI	80% CI		
Performance	beta_0	-0.417	[-2.290, 1.482]	[-1.653, 0.815]	0.371	[-1.502, 2.232]	[-0.872, 1.594]	0.442	0.558
	beta_elev	0.029	[-0.031, 0.090]	[-0.010, 0.070]	0.001	[-0.058, 0.061]	[-0.038, 0.041]	0.522	0.478
	beta_elev_mu	0.034	[-0.029, 0.094]	[-0.006, 0.073]	0.029	[-0.028, 0.087]	[-0.009, 0.066]	0.751	0.249
R:S ratio	beta_0	0.277	[-1.655, 2.169]	[-0.956, 1.497]	0.404	[-1.514, 2.276]	[-0.857, 1.647]	0.527	0.473
	beta_elev	-0.011	[-0.072, 0.051]	[-0.051, 0.029]	-0.019	[-0.079, 0.042]	[-0.059, 0.021]	0.577	0.423
	beta_elev_mu	0.065	[0.008, 0.121]	[0.027, 0.102]	0.061	[0.002, 0.121]	[0.022, 0.100]	0.968	0.032

	Performance			R:S ratio		
	Mean	95% CI	80% CI	Mean	95% CI	80% CI
heritability	0.291	[0.186, 0.428]	[0.219, 0.371]	0.198	[0.120, 0.310]	[0.141, 0.264]
sd_individual	0.625	[0.576, 0.680]	[0.592, 0.660]	0.768	[0.708, 0.833]	[0.728, 0.810]
sd_mother	0.401	[0.303, 0.534]	[0.334, 0.476]	0.381	[0.286, 0.511]	[0.313, 0.456]
sd_bin	0.723	[0.537, 0.992]	[0.590, 0.871]	0.698	[0.513, 0.965]	[0.563, 0.850]