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The macroevolution of functional leaf traits in the genus *Coffea*

Understanding adaptive patterns across continental Africa and the West Indian Ocean Islands

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Preface

This thesis summarizes two semesters of research, to fulfill the requirements of graduation for the Master of Biology at KU Leuven. During this process, I have learned new technical, professional, and communicative skills that I did not fully anticipate. I have gained confidence in performing statistical analyses, scientific writing, and most importantly, figuring things out on my own. The full process from start to finish has been challenging, and I could not have predicted the extent of the learning process it has encompassed.

I would like to express my gratitude to my tutor, Jonas Depecker, my cosupervisor, Filip Vandelook, and my supervisor, Olivier Honnay, for their patient guidance throughout this endeavor. Their advice and expertise provided me with the needed tools to complete this thesis.

Thanks should also go to my classmates and friends, whose moral support and feedback provided me with the much needed confidence to persevere. I am grateful to them for listening to me talk about this thesis, but also for sometimes stopping me from doing so.

Lastly, I would like to acknowledge my parents, brothers, and girlfriend. Their continuous encouragement allowed me to stay motivated and put things into perspective.

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Summary

The importance of functional diversity is being increasingly recognized in ecological and evolutionary research. An organism's functional traits can impact its performance in its environment, and can therefore be expected to (co)vary along environmental gradients. Additionally, close relatives of domesticated plant species serve as an important gene pool for crop improvement. In this study, we studied the diversity and evolution of six functional leaf traits in the coffee genus, *Coffea* L. Coffee is of immense societal and economic value worldwide, yet the evolutionary processes involved in shaping its diversity remain poorly understood. The six leaf traits examined in this study were stomatal density, leaf area, Specific Leaf Area (SLA), stomatal length, stomatal width, and pore width. Across continental Africa and the West Indian Ocean Islands, we tested for correlations among traits, and between traits and climate using phylogenetic Generalized Least Squares regressions. We also compared different models of continuous trait evolution across the phylogeny of *Coffea*, in order to investigate the evolutionary trajectories of the studied traits.

Our results showed that the trait variation between species is substantial, and is generally larger than within species. A clear trade-off exists between stomatal size and stomatal density. However, this trade-off axis most likely represents spatial constraints rather than a range of adaptive strategies. In contrast, we have shown that leaf area has evolved adaptively as a function of precipitation, with larger-leaved species generally occurring in more humid rainforests closer to the equator. Stomatal density and possibly stomatal size increase with leaf area, suggesting that species in environments with abundant precipitation have a higher total pore area to maximize carbon uptake. Our analyses suggested that SLA increases with temperature and does not respond significantly to precipitation, indicating that leaf area and SLA regulate different aspects of environmental adaptation.

Evolutionary model comparisons indicated that stabilizing selection is the main driver of the present diversity in the studied traits across *Coffea*. Absence of phylogenetic signal and high rates of trait change towards optimal trait values further supported this conclusion. This implies that leaf trait divergence in the genus is constrained by selection towards an intermediate optimum, and that closely related species thus remain ecologically more similar to each other. We have shown that leaf traits are capable of rapid evolution towards new adaptive peaks, when exposed to novel selective pressures. Overall, the genus seems to have diverged towards higher leaf investment and higher densities of small stomata over time. The conclusions of this study contribute to the knowledge on leaf trait variation across the *Coffea* genus, and its adaptive significance. Our results can help inform prospective avenues for crop improvement, in order to support more effective and sustainable coffee production worldwide.

Symbols and Acronyms

α Strength of selection towards the optimum in an Ornstein-Uhlenbeck model of evolution.

λ Pagel's lambda.

ρ Spearman correlation coefficient.

σ^2 Brownian rate of character evolution.

θ Optimum trait value in an Ornstein-Uhlenbeck model of evolution.

$t_{1/2}$ Phylogenetic half-life.

ACDC Accelerating/Decelerating.

AFR African.

AIC Akaike Information Criterion.

AICc small-sample corrected Akaike Information Criterion.

ANCOVA Analysis of Covariance.

ANOVA Analysis of Variance.

BIC Bayesian Information Criterion.

BM Brownian Motion.

CI Confidence Interval.

EB Early Burst.

EC Eu-coffee.

LES Leaf Economics Spectrum.

LMA Leaf Mass per Area.

MCMC Markov Chain Monte Carlo.

ML Maximum Likelihood.

OU Ornstein-Uhlenbeck.

OU1 OU model with a single optimal trait value.

OUM OU models with multiple optimal trait values.

PC Principal Component.

PCA Principal Component Analysis.

PCM Phylogenetic Comparative Method.

pGLS phylogenetic Generalized Least Squares.

SE Standard Error.

SLA Specific Leaf Area.

WIOI West Indian Ocean Islands.

WUE Water Use Efficiency.

XC Xeno-coffee.

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Chapter 1

Introduction

1.1 Leaf trait data

Leaf traits as proxies for functional processes in plants

Biodiversity is widely known to be an important element of the health and functioning of ecosystems (Chapin et al., 1997; Duffy, 2009). In recent decades, though, the concept of biodiversity has increasingly come to be interpreted not just as the diversity of species, but also as the diversity of the functional and structural characteristics these taxa exhibit (Tilman, 2001). Such an interpretation provides much more information about why species live where they do, and how they interact with one another (Cadotte et al., 2015; McGill et al., 2006). This shift in thinking gave rise to the functional diversity concept, which refers to the diversity of organismal traits that can have an impact on the functioning of an ecosystem (Tilman, 2001).

Trait-based research has become progressively more prominent since the turn of the century (Cadotte et al., 2015; McGill et al., 2006; Tilman, 2001; Violle et al., 2007). Though multiple definitions exist (Dawson et al., 2021), an organismal trait is defined by McGill et al. (2006) as "a well-defined, measurable property of organisms, usually measured at the individual level and used comparatively across species". Traits that have the potential to significantly influence the performance or fitness of an organism in a certain environmental niche, or can affect the ecosystem surrounding the organism, are often referred to as functional traits (McGill et al., 2006; Reich et al., 2003) (for an in-depth discussion of the term "functional trait", see Violle et al. (2007)). These traits can respond to environmental cues, meaning that they can impact certain ecological functions of the organism by responding to variation in the environment (Klápště et al., 2021).

Phenotypic traits in leaves are often a strong predictor of a plant's performance in a certain habitat (Poorter and Bongers, 2006; Violle et al., 2007). Several leaf traits can reflect adaptation or maladaptation of a plant to the environment surrounding it. For example, increasing moisture levels are associated with darker, larger leaves, with succulent, lighter-colored leaves occurring in drier climates (Wang et al., 2022). By growing thick leaves, the area to mass ratio of the leaf is reduced in order to mitigate water loss via transpiration (Vendramini et al., 2002). Leaf functional traits are not necessarily morphological (*e.g.*, leaf area, dry mass) but

can also be physiological (*e.g.*, net assimilation rate, light compensation point) (Poorter, 1999) or phenological (*e.g.*, deciduousness, seasonality of germination) (Mcintyre et al., 1999; Violle et al., 2007). Some examples of leaf traits that are commonly used for interpreting plant life strategies include leaf lifespan, Specific Leaf Area (SLA) (defined as the ratio of leaf area to leaf dry mass), Leaf Mass per Area (LMA) (the inverse of SLA) or photosynthetic capacity (Reich, 2014; Wright et al., 2004).

These different leaf traits are not expressed in isolation: certain functional traits are often correlated with each other and covary with the environment. These trait correlations reflect an individual plant's strategy for survival and reproduction (Reich, 2014). A general framework summarizing some of the most universal trait combinations is the Leaf Economics Spectrum (LES) (Figure 1.1), described by Wright et al. (2004). The LES represents a continuum of life history strategies, explaining close to three quarters of global variation in six key leaf traits. At one end of the spectrum are slow-growing, long-lived, and dense leaves, with a low photosynthetic rate. The other end represents short-lived, fast-growing leaves that require little investment, and have a higher SLA and photosynthetic rate (Reich, 2014; Wright et al., 2004). The LES framework was later expanded by Reich (2014) to encompass other plant resources such as water, as well as traits in stems and roots. The overarching "fast-slow" continuum of strategies can even help explain ecological processes at community and ecosystem scales (Reich, 2014). The LES illustrates the trade-offs between different strategies: a balance needs to be found between short-term gain and long-term survival, which is then reflected in leaf trait variation (Poorter and Bongers, 2006).

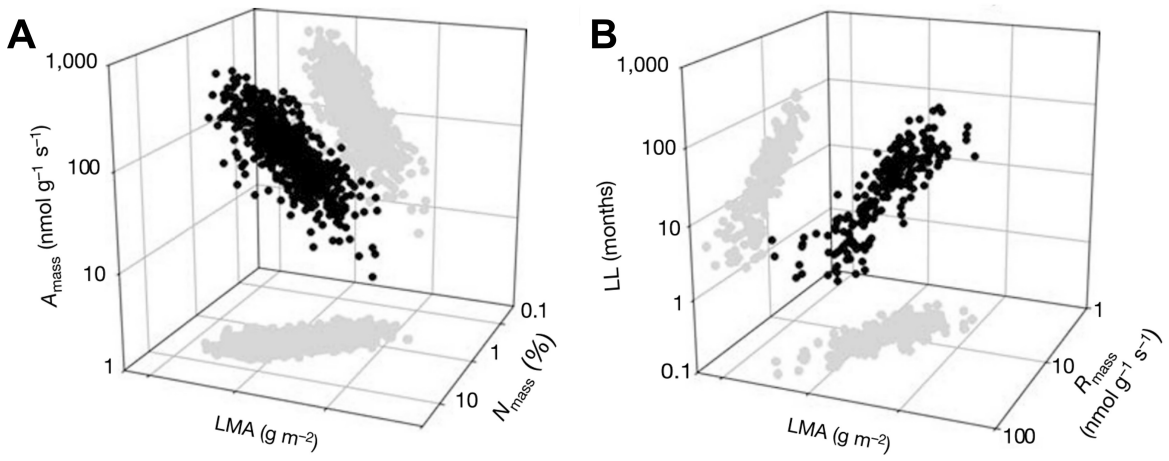


Figure 1.1: A visualization of the Leaf Economics Spectrum (adapted from Wright et al. (2004)). **A**: Relationship between A_{mass}, LMA and N_{mass}; **B**: Relationship between LL, LMA and R_{mass}.

The trade-offs in the LES demonstrate that evolutionary adaptation is not without limits: plants cannot express opposing strategies simultaneously. Also, whether or not a plant has the capacity to adapt to its environment depends on the patterns of covariation exhibited between traits (Klápště et al., 2021). The degree to which the covarying traits are coherent and coordinated is often referred to as "phenotypic integration" (Armbruster et al., 2014; Klápště et al., 2021; Magwene, 2008; Wagner and Altenberg, 1996). A group of traits that is strongly integrated with each other is called a functional unit, or module (Klápště et al., 2021). The counterpart of phenotypic integration is modularity, which indicates how strongly

different functional units of traits vary independently from each other, as well as how integrated the traits are within and between functional units (Klápště et al., 2021; Wagner et al., 2007; Wagner and Altenberg, 1996). Alternatively, modularity could be described as the amount of pleiotropic effects among traits that serve different functions: a more modular phenotype would be one where pleiotropic effects occur among traits that are part of the same functional unit (Klápště et al., 2021; Klingenberg, 2008; Wagner and Altenberg, 1996; Wagner et al., 2007). This integration can prevent independent evolution of integrated traits if selection occurs in a different direction than the correlation of the traits. On the other hand, integration can accelerate evolution if trait groups are highly modular; if traits can evolve as a functional unit, they can respond more effectively to new selective forces (Klápště et al., 2021; Pigliucci, 2003). Correlations between traits could be purely phenotypic, but generally have a genetic basis: possible causes of these genetic correlations are pleiotropy or linkage disequilibrium (Smith et al., 1985).

Collectively, constraints on adaptation are often referred to as phylogenetic inertia. The term "phylogenetic inertia" is defined by Hansen et al. (2008) as "a resistance to or slowness in the adaptation to a specific optimum". Generally, it is used to indicate a constraint on adaptation due to the evolutionary history of a lineage, leading to imperfect adaptation of a trait to its current environment (Blomberg and Garland, 2002; Hansen and Orzack, 2005). Any maladaptation in a trait will therefore be correlated in related species (Hansen et al., 2008). Inertia can have a variety of causes, including limited genetic variation or conflicting selection pressures over time (Hansen and Orzack, 2005). If the rate of trait evolution cannot keep up with the rate of environmental change, phylogenetic inertia can be a cause of niche conservatism (Cooper et al., 2010).

Niche conservatism, or the tendency for species to retain aspects of their ancestral fundamental niche over evolutionary time (Wiens and Graham, 2005), is an important process in evolution that peaks the interest of many researchers (Cooper et al., 2010). With strong niche conservatism, more related species will remain ecologically more similar to each other (Losos, 2008). The occurrence of niche conservatism suggests that divergence among related species is constrained, thus causing ancestral traits to be retained. The retention of an ancestral trait does not necessarily mean that this trait is maladaptive: it is possible that the trait is simply maintained by stabilizing selection (Hansen, 1997). Aside from phylogenetic inertia or stabilizing selection, some degree of niche conservatism can, for example, be caused by a pure drift process (Cooper et al., 2010).

Leaf traits and the environment

Due to the predictive value of leaf trait observations for inferring plant performance in a given habitat, certain traits might be logically expected to vary geographically, adapted to the local climate (Poorter and Bongers, 2006; Violle et al., 2007). In predicted future climate scenarios, Water Use Efficiency (WUE) (*i.e.*, grams of CO_2 assimilated per gram of water lost) will likely be an essential target for optimization in crops (Flexas, 2016). Functional leaf traits, in a climate context, can generally be assumed to maximize WUE (López et al., 2021; Parkhurst and Loucks, 1972; Shipley et al., 2005; Farquhar et al., 2002). This assumption is reflected in the expected correlations of the traits with climatic variables.

Following this optimality-based rationale, leaf size should theoretically increase with rainfall and decrease with temperature. However, temperature and precipitation tend to interact in their effects on leaf size (Wright et al., 2017). Leaves tend to be larger in hot and wet climates, as is the case in the tropics. Small leaves predominate in hot but arid conditions, as well as cooler regions at higher altitudes or latitudes. The effect of temperature on leaf size thus depends strongly on precipitation levels (Wright et al., 2017). The same logic can be followed for SLA, which should be lower when desiccation risk is higher. Morphologically, leaves have also been found to become more elongated with higher temperatures (Peppe et al., 2011). A longer, narrower leaf shape likely allows for a better emission of heat in hot environments (Givnish, 1984; Peppe et al., 2011).

When studying stomata across different climates, a number of traits can be considered. Stomatal density (*i.e.*, number of stomata per unit area), for example, is closely linked to WUE (Xu and Zhou, 2008). Multiple studies have found that stomatal density tends to increase with drought stress, due to the larger leaf area when water is not limiting (McCree and Davis, 1974; Xu and Zhou, 2008). In warm, dry environments, plants are thus expected to have more stomata per unit of area. Aside from stomatal density, the size of stomata is important to consider: together, these two traits determine the stomatal conductance, *i.e.*, the rate of CO_2 uptake and transpiration through the stomata (Franks and Beerling, 2009; Franks and Farquhar, 2001; Jordan et al., 2015). Stomatal conductance is negatively correlated with WUE (Drake et al., 2013), so we would expect more, smaller stomata in dryer, hotter environments, where transpiration needs to be minimized (Bertolino et al., 2019; Doheny-Adams et al., 2012; Franks and Beerling, 2009). Indeed, plants grown under drought stress have been found to have smaller stomata than plants grown under adequate moisture conditions (Spence et al., 1986). Leaves with smaller stomata have been shown to have a higher maximum CO_2 absorption capacity for the same total pore area (Brodribb et al., 2013; Franks and Beerling, 2009). Additionally, with smaller stomata, stomatal density can increase allowing for a great increase in CO_2 exchange (Brodribb et al., 2013). However, this is inevitably paired with greater water loss. Overall, the importance of climate as an explanation of stomatal size remains unclear and requires further investigation (Jordan et al., 2015).

Aforementioned expectations of trait correlations with climate arise merely from global patterns or observations in specific taxa. Generalizations must therefore be applied with caution, since these patterns are not necessarily applicable in all regions or lineages. Previous research has indicated that correlations between climate variables and plant traits are generally very weak, due to the large amount of trait variation present between coexisting species (Moles et al., 2005; Vandelook et al., 2012; Wright et al., 2004). Nonetheless, these general associations of leaf traits with climate can serve as rules of thumb for interpretations of evolutionary and (paleo)climatic studies (Peppe et al., 2011).

Studying leaf trait evolution

The inability for an individual plant to migrate away from its germination site emphasizes the importance of plant responses to their environment (Schlichting, 1986). An organism can respond phenotypically to its environment in two major ways (Mitchell and Bakker, 2014). One possibility is phenotypic plasticity, where a single genotype, within its lifetime, can present different phenotypes in response to a change in its (biotic or abiotic) environment (Bradshaw, 1965; Pigliucci, 2001). For these phenotypes to be inherited across generations, however,

genetic changes are necessary. Over longer timescales, this leads to the second type of response to the environment: adaptation via evolutionary change (Bradshaw, 1965; Joshi et al., 2001).

Many plants have evolved notable plastic responses to short-term variation in their surroundings. For example, multiple species have been found to bias biomass allocation toward roots when water and nutrients are limited, or toward stems and leaves when light is the factor limiting growth (Brouwer, 1963; Callaway et al., 2003; Chapin, 1980; Yan et al., 2019). Aside from plastic responses, which occur within an individual's lifetime, adaptation across generations is vital for long-term survival of a genetic lineage (Davis and Shaw, 2001; Östergren et al., 2021; Templeton et al., 2001). Therefore, variation in functional traits is essential to respond to stochastic variation in environmental variables, as well as to allow selection, and thus possibly adaptation to new environments (Fisher, 1930).

To study long-term evolutionary processes across phylogenies, experimental approaches are not readily available. Aside from specimens in the fossil record, we cannot directly compare extant taxa to their ancestors. Therefore, most researchers turn to Phylogenetic Comparative Methods (PCMs) to test hypotheses on long-term processes (Cooper et al., 2010; Felsenstein, 1985; Freckleton, 2009; Smith, 1978). These methods are particularly useful for answering questions about macroevolutionary processes. Determining the traits of a common ancestor, how traits influence each other's evolution, or how fast groups of species are evolving or diversifying are all questions that can be investigated with PCMs (Martins and Hansen, 1997; O'meara, 2012). For instance, Vieu et al. (2021) used a PCM framework to examine trait evolution in the *Macrocarpaea* genus. Their results indicated that adaptive divergence was unlikely to have played a role in the radiation of the genus, and that geographical processes were more important in facilitating speciation. The study also exemplifies the fact that, despite their relatedness, species within the same genus can fulfill very different functional roles in their environment, depending on their unique trait combinations (Vieu et al., 2021).

Before hypotheses about evolutionary processes can be tested, though, models are first required to adequately predict the macroevolutionary patterns expected with hypothetical microevolutionary change (Hansen and Martins, 1996). Multiple types of models have been described, encompassing different types of trait evolution. Here, we will focus on the evolution of continuous traits.

Models of continuous trait evolution

The simplest model of an evolutionary process commonly used in comparative analyses is a Brownian Motion (BM) model, where traits are assumed to change randomly over time at a given rate (Meireles et al., 2020; Revell et al., 2008). BM is essentially a constant-variance random-walk model, and can be used to describe a host of stochastic processes, including the evolution of continuous traits (Freckleton et al., 2002). The general principle is that for a given trait X, the change over time in a BM model of evolution can be described by Equation 1.1:

$$dX = \sigma^2 dt \tag{1.1}$$

In each infinitesimal time step dt, the trait value evolves with a mean change of 0 and a constant variance of σ^2. The parameter σ^2 denotes the Brownian rate of character evolution (*i.e.*, the magnitude of trait change in each time step) and t denotes the time during which the variation occurs (Flores et al., 2014; Hansen, 1997; Pagel, 1999). Brownian Motion is by definition a Markovian process, meaning that the process is temporally uncorrelated and only depends on the current state of the trait at each time point (Hansen and Martins, 1996). Thus, BM assumes that trait values among species are correlated proportionally to their shared ancestry. The detection of a BM model of evolution in empirical data could suggest random genetic drift, or, for example, natural selection fluctuating in direction and intensity through evolutionary time (Losos, 2008; O'Meara et al., 2006).

However, BM also implicitly assumes that trait values can change infinitely, given enough evolutionary time. Limits on evolution can for example be imposed by a lack of genetic variance (Blows and Hoffmann, 2005), trade-offs or genetic correlations (Reznick et al., 2000; Sgrò and Hoffmann, 2004), or natural selection on multiple traits (Fisher, 1930; Orr, 2000). In scenarios where there is stabilizing selection toward a given optimal trait value, Ornstein-Uhlenbeck (OU) models can be applied (Beaulieu et al., 2012; Hansen, 1997; Butler and King, 2004). OU processes represent BM under friction. These models are essentially an extension of BM where traits vary randomly, but with a certain attraction to an intermediate optimum. This attraction is modelled with the inclusion of a mean-reverting process in the trait value. This is reflected in a mathematical description of OU models, as shown in Equation 1.2 (Butler and King, 2004):

$$dX_t = \alpha(\theta - X_t)dt + \sigma dB_t \tag{1.2}$$

In this equation, θ represents the optimum value for trait X; α denotes the strength of selection towards this optimum, *i.e.*, the strength of mean-reversion; σdB_t represents the unmodified BM process (Beaulieu et al., 2012; Butler and King, 2004). Thus, one term in this model is a pull towards an optimum value that increases linearly in strength with the distance from this optimum, and the other is a stochastic BM process (Beaulieu et al., 2012; Hansen, 1997; Hansen et al., 2008). Interpretation of the α parameter is not straightforward, though, and the more intuitive "phylogenetic half-life" $t_{1/2}$ is often reported (Hansen, 1997; Pan et al., 2014; Vandelook et al., 2018). This half-life is calculated as in Equation 1.3:

$$t_{1/2} = log_e(2)/\alpha \tag{1.3}$$

Phylogenetic half-life represents the time required for a trait to move halfway from its ancestral state to the optimum value, and (like α) must always be interpreted in relation to the full root-to-tip length of the phylogeny (Hansen, 1997). Ornstein-Uhlenbeck models predict that most of the trait variation will already be present in young clades, despite their recent establishment (Harmon et al., 2010). Therefore, young clades will contain about the same amount of trait variation as older groups of taxa under an OU model.

A third type of evolutionary change is described in Accelerating/Decelerating (ACDC) (Blomberg et al., 2003) or Early Burst (EB) (Harmon et al., 2010) models. These models represent BM evolution, but with a rate that can increase or decrease over time. The slowing rate of evolution in EB models thus represents the gradual filling of niche space over

time, as would be expected during adaptive radiations (Blomberg et al., 2003; Freckleton and Harvey, 2006; Harmon et al., 2010). Under an EB model of evolution, young clades are predicted to contain little variation relative to older clades due to the drop in evolutionary rates over time (Harmon et al., 2010). An EB model can be described by allowing the Brownian rate of evolution σ^2 to change exponentially with time, according to a parameter g (Blomberg et al., 2003; Silvestro et al., 2015). The rate of evolution decreases over time if $g < 1$, and increases when $g > 1$ (Blomberg et al., 2003).

Phylogenetic Comparative Methods (PCMs)

When attempting to infer evolutionary history from comparative data, it is important to account for the phylogenetic relatedness of the species being compared. Since the species are part of the same hierarchical phylogeny, they often cannot be regarded as independent samples (Clutton-Brock and Harvey, 1977; Felsenstein, 1985). Consequently, any traditional statistical tests performed on the data will be compromised, p-values will not be reliable and Type I error rates (*i.e.*, false positives) will become inflated (Felsenstein, 1985; Martins and Garland, 1991).

The non-independence of trait data across related species is often described as the presence of phylogenetic signal, which refers to the degree to which a certain trait is similar in related taxa (Ackerly, 2009; Blomberg and Garland, 2002; Revell et al., 2008). If the trait is more similar in more related taxa, there is a stronger phylogenetic signal. Evolution under different evolutionary models will lead to different outcomes of phylogenetic signal in the trait data. In a BM evolutionary process, phylogenetic signal will intrinsically arise, precisely because trait variation is dependent on time spent evolving independently (Blomberg and Garland, 2002; Losos, 2008). Evolution according to an OU process with a single optimum will, as a rule, reduce phylogenetic signal in the trait values (Blomberg et al., 2003; Felsenstein, 1988). In EB models, a rapidly decelerating evolutionary rate should lead to relatively strong phylogenetic signal in trait data (Blomberg et al., 2003; Harmon et al., 2010).

Multiple metrics have been proposed to quantify the strength of phylogenetic signal in any given trait (Blomberg et al., 2003; Pagel, 1999). One of the most commonly used quantifiers is Pagel's lambda (λ) (Pagel, 1999), which normally ranges from 0 to 1. A λ-value of 1 indicates that traits among species are as similar as would be expected from their phylogenetic relationships, assuming a BM model of evolution. A high λ-value thus indicates that trait evolution occurred in a manner similar to Brownian Motion. $\lambda = 0$, on the other hand, implies that there is no phylogenetic structure in the data or that the data can be deemed phylogenetically independent (Cooper et al., 2010; Freckleton et al., 2002; Vandelook et al., 2012). If λ does not differ significantly from 0, correction for phylogenetic relatedness becomes irrelevant (Vandelook et al., 2012). Simulations have shown that λ is a statistically powerful metric of phylogenetic signal in species data (Freckleton et al., 2002).

The field of PCMs has gained popularity in recent decades, and many different statistical approaches have been developed to work around the issue of phylogenetic non-independence (O'meara, 2012). Since the development of the independent contrasts method (Felsenstein, 1985) and the phylogenetic regression (Grafen, 1989), researchers have developed phylogenetic alternatives for many traditional statistical methods. Some of the most commonly applied methods include phylogenetic Analysis of Variance (ANOVA) and Analysis of Covariance (ANCOVA) (Garland et al., 1993), phylogenetic Generalized Least Squares (pGLS) regres-

sion (Grafen, 1989; Martins and Hansen, 1997) and phylogenetic PCA (Revell, 2009). These PCMs have been constantly built upon, for example to quantify uncertainty in parameter estimates (Boettiger et al., 2012), fit high-dimensional data sets (Clavel et al., 2019), or support multivariate analyses (Clavel and Morlon, 2020).

Previous research in leaf trait evolution

Leaf trait variation and evolution have received quite some interest from researchers in the past. Examples of leaf trait studies include studies within (Dubberstein et al., 2021) or between a few species (Dutra Giles et al., 2019), within families (Onstein et al., 2016), or even across all vascular plants (Flores et al., 2014).

In a study across over 5000 vascular plant species, Flores et al. (2014) investigated the evolutionary history of Leaf Mass per Area (LMA). They determined that LMA evolved under weak stabilizing selection, with different optima in different clades and selection against extreme phenotypes. Also, woody taxa were reported to evolve at lower rates and exhibit more niche conservatism than herbaceous species. Their study provides an example of evidence for the existence of multiple evolutionary patterns across species, as well as insight into the evolution of ecological strategies in plants (Flores et al., 2014).

At the family level, Onstein et al. (2016) studied the evolutionary patterns of leaf area, leaf shape, and sclerophylly, as well as climatic niches, across the Proteaceae family. Using pGLS regressions and different models of quantitative trait evolution, they concluded that specific leaf adaptations may have evolved in order to adapt to different climates. Divergent selection towards different trait optima in different environments likely triggered an evolutionary radiation within the Proteaceae family (Onstein et al., 2016).

Studies of leaf traits have been performed at the intrageneric and intraspecific level, in order to detect genotypes that might be useful for preservation and agricultural purposes (Dubberstein et al., 2021; Dutra Giles et al., 2019). Dutra Giles et al. (2019) analyzed divergence in genotypes of the two main commercial coffee species (*Coffea arabica* L. and *C. canephora* Pierre ex A. Froehner), based on a set of morpho-anatomical leaf traits. They detected significant divergence between genotypes, showing potential for future breeding endeavors (Dutra Giles et al., 2019). Similarly, Dubberstein et al. (2021) were able to identify a number of genotypes with highly suitable traits for breeding purposes in *C. canephora* by inspecting variation in stomatal traits. Their results emphasize the need for diversity in agricultural species to be able to adapt to different environmental conditions (Dubberstein et al., 2021).

1.2 The coffee genus: *Coffea* L. (Rubiaceae)

A general description

Coffea L. is a genus of eudicots in the Lamiid clade, a monophyletic group within the Asterids (APG III, 2009; Bremer et al., 2002). The genus is situated in the Coffeeae tribe of the Rubiaceae subfamily Ixoroideae, within the order Gentianales. The Coffeeae tribe contains ten other genera besides *Coffea*, including for example *Belonophora*, *Calycosiphonia*, and *Tricalysia* (Davis et al., 2007). *Coffea* most likely originated in Africa, Asia or the Arabian peninsula and now occurs naturally in tropical and southern Africa, and parts of tropical Asia (Hamon et al., 2017).

In 2011, the genus *Psilanthus* Hook.f. was subsumed into *Coffea*, after originally being classified as *Coffea*'s closest relative (Davis et al., 2007, 2011; Robbrecht and Manen, 2006). Despite some morphological differences, molecular markers and morphological data supported the inclusion of *Psilanthus* into *Coffea*, leading to the addition of 20 former *Psilanthus* species in the genus. With this inclusion, *Coffea* currently comprises a total of 124 known species (Davis et al., 2011; Hamon et al., 2017).

Depending on the species, plants in the *Coffea* genus can range from bushes to small trees, generally residing in tropical evergreen forest understories (Davis et al., 2006; Herrera and Lambot, 2017). The simple leaves are oppositely placed along the stems, and are generally smooth, waxy, and elliptical, with marked venation (Petruzzello, 2021). The leaves can vary among species in size and shape. For example, *C. liberica* Hiern leaves can range up to 42 cm long and 20cm wide (Cheney, 1925; Davis et al., 2022). The leaves of *C. mufindiensis*, on the other hand, are much smaller, up to 7 cm in length (Bridson, 1982). Some species, like *C. humilis*, have more elongated leaves than the other members of the genus. Leaf edges can range from entirely smooth in some species, to wavy in others (Gava Ferrão et al., 2019; Bridson, 1982).

The variety of *Coffea* species

Hamon et al. (2017) used genotyping-by-sequencing to produce the first well-resolved, robust phylogeny of the *Coffea* genus. They used parsimony, maximum likelihood, and Bayesian inference methods to reconstruct the phylogenetic structure of the 124 known *Coffea* species with high branch support (see Figure B.1 in Appendix B for a pruned version of this phylogeny). Their phylogeny identified two major clades within the coffee genus: Xeno-coffee (XC) and Eu-coffee (EC). XC comprises all former *Psilanthus* species as well as *C. rhamnifolia*, while EC contains all other *Coffea* species. The XC clade contains two basal species (*C. rhamnifolia* and *C. neoleroyi*) and two subclades: a West and Central African subclade, and an Asian (Indian and Indonesian) subclade. The EC clade contains the majority of *Coffea* species and has a basal branch occupied by *C. charrieriana*, followed by two major subclades which diversified independently in Africa (AFR subclade) and on Western Indian Ocean Islands (WIOI subclade) (Hamon et al., 2017). Aside from *Coffea* species, Hamon et al. (2017) included five related species as outgroups in the phylogenetic reconstruction: *Bertiera iturensis*, *Tricalysia congesta*, *Belonophora coriacea*, *Argocoffeopsis eketensis*, and *Calycosiphonia spathicalyx*. All of these outgroup species are situated in the Coffeeae tribe, except *Bertiera iturensis*, which belongs to a different tribe within the Ixoroideae subfamily.

All of the 124 known *Coffea* species are diploid, except for the allotetraploid hybrid species *C. arabica* (Carvalho, 1952; Gava Ferrão et al., 2019). *C. arabica* is predominantly self-pollinating, as opposed to the self-incompatible widespread species *C. canephora* and *C. liberica* (Gomez et al., 2009). Besides *C. arabica*, the only other species currently known to be self-compatible are *C. heterocalyx* and *C. anthonyi* (Anthony et al., 2010). *C. canephora* and *C. liberica* have overlapping geographical ranges and have the widest natural distributions out of all coffee species: both species occur across central Africa, stretching from Guinea east to Uganda, and south to Angola (Davis et al., 2006; Gomez et al., 2009; N'Diaye et al., 2005). Most coffee species that are widespread across large areas of the African continent (*e.g.*, *C. canephora*, *C. liberica*, *C. eugenioides*) inhabit humid habitats, such as evergreen tropical forests or forest patches along rivers or wetlands (Herrera and Lambot, 2017). Nonetheless, some species can also be found in dryer shrublands, or deciduous forests with a dry season (Herrera and Lambot, 2017; Maurin et al., 2007). Most species, however, have a relatively narrow geographic distribution (Davis et al., 2006). This points to the possibility that the current wide range of *C. canephora* and *C. liberica* is significantly larger than it would have been without introduction for consumption, the effects of which are hard to discern from indigenous distributions (Davis et al., 2006).

A natural barrier divides the forests of West and Central Africa. An arid region in and around Togo and Benin, named the Dahomey Gap, forms a barrier for the dispersal of many coffee species between Upper-Guinean and Guineo-Congolian rainforests (Cubry et al., 2013). This savannah-like region is thought to have become established around 4000 years BP (Salzmann and Hoelzmann, 2005). At least for *C. canephora* and *C. liberica*, the Dahomey Gap has been shown to have influenced the genotype diversity of the species (Berthaud, 1986; Gomez et al., 2009). Phylogenetic evidence also indicates that speciation within the *Coffea* genus has been influenced by the presence of the Dahomey Gap (Maurin et al., 2007).

The majority of the species in the WIOI subclade occur on the island of Madagascar, though some species inhabit Comoros and the Mascarenes as well (Hamon et al., 2017). Genetic studies have indicated rapid and radial speciation on Madagascar, indicative of an adaptive radiation (Anthony et al., 2010; Davis et al., 2006). For example, *C. ratsimamangae* is endemic to north-eastern Madagascar and occurs only in seasonally dry evergreen forests between 0 and 400m of altitude, with admixture of deciduous species (Davis et al., 2006; Davis and Rakotonasolo, 2001). In contrast, *C. kianjavatensis* occurs only in humid evergreen forests, between 300 and 500m of altitude on the eastern side of Madagascar (Davis et al., 2006).

As of December 2022, the IUCN Red List of Threatened Species classifies 75 *Coffea* species as at risk for extinction, including 22 Vulnerable (VU), 40 Endangered (EN) and 13 Critically Endangered (CR) species (IUCN, 2022). This amounts to over 60% of all *Coffea* species and does not include another 14 species which are currently classified as Data Deficient (DD). Additionally, 45% of coffee species are not conserved *ex situ* in any germplasm collections (Davis et al., 2019). Out of the 13 species classified as CR, seven are currently known to be decreasing in abundance; the status of the other six species is not known (IUCN, 2022).

The economic and societal value of coffee

Each year, close to 10 million tons of coffee are consumed worldwide (International Coffee Organization, 2021). Over half (51%) of this coffee is consumed in Europe and North America alone, illustrating the divide between the global North and the global South in the coffee industry. Coffee is grown commercially across large parts of the world, but the largest producers of coffee are Brazil, Vietnam, and Colombia (International Coffee Organization, 2021). Suitable climates for coffee plantations lie between the Tropics of Cancer and Capricorn, approximately from 23°N to 23°S (Consonni et al., 2012). This band around the equator is often referred to as the "Bean Belt" due to the humid climate and nutrient-rich soil that provide favorable growing conditions for coffee cultivation (National Coffee Association USA, nd). It is estimated that around 25 million smallholder coffee producers account for 80 percent of the global coffee production (FAO, 2018), though other sources have provided lower estimates around 12.5 million (Rushton, 2019). Coffee is thus a valuable crop in Oceania, Asia, Africa, and the Americas, providing a livelihood to millions of people involved in the growing, production, and processing of coffee (Harvey et al., 2021). Aside from its contribution to farmers' livelihoods, coffee is widely traded as a commodity on global markets, making it an important product in the global economy (OECD, 2022).

Despite the large amount of species in the genus, only a few are actually grown commercially: *C. arabica* (Arabica coffee), *C. canephora* (Robusta coffee), and rarely *C. liberica* (Liberica or Excelsa coffee). Arabica coffee, which is a hybrid between *C. canephora* and *C. eugenioides*, makes up the majority of global production (60%) (International Coffee Organization, 2021). At 40% of global coffee production, the rest of the market is almost entirely made up of Robusta coffee (International Coffee Organization, 2021). Liberica coffee currently constitutes an almost negligible percentage of global coffee production, but is nonetheless produced and consumed locally in the Philippines and parts of south-east Asia (Philippine Coffee Board, 2018; Santos and Cao, 2020). Liberica was once a widespread commercial crop, but declined due to a variety of reasons (including the rapid dissemination of *C. canephora*) (Davis et al., 2022). Recently though, Liberica coffee has been gaining interest across global coffee markets and could potentially re-emerge as a commercially grown species (Davis et al., 2022).

Threats to global coffee production

One of the largest threats to coffee production worldwide is the advance of *Hemileia vastatrix*, a fungus that causes a disease called coffee leaf rust. The fungus invades the leaves of the coffee plant and reduces the plant's capacity for photosynthesis, leading to yield reductions of up to 35% (Talhinhas et al., 2017). The reproduction of the fungus is aided by high levels of precipitation that characterize the habitat of commercial coffee species (McCook, 2006). The fungus has had extensive consequences for coffee production worldwide, even leading to the abandonment of coffee cultivation altogether in Sri Lanka in the late 1800's (McCook, 2006). Even today, the fungus is still responsible for an estimated 1-2 billion US dollars in economic losses each year (McCook, 2006).

Aside from biotic risks, the increased incidence of drought in the changing global climate has caused production deficits in many coffee-growing regions (Davis et al., 2022; International Coffee Organization, 2022). Temperature, soil moisture, and humidity in the air also affect coffee productivity, and future climate conditions are projected to further impact yields going

forward (Kath et al., 2022). Furthermore, many regions where coffee is currently grown will likely become unsuitable for cultivation: in Ethiopia, for example, conditions in 39 to 59% of current coffee crop regions are predicted to become inadequate for coffee growing by the end of the century (Moat et al., 2017). In Nicaragua, over 90% of areas suitable for Arabica cultivation are predicted to show decreases in suitability, particularly at lower altitudes (Läderach et al., 2017).

Currently, the strategy that is considered most viable to combat the detrimental effects of leaf rust (Talhinhas et al., 2017) and climate change (Davis et al., 2021, 2022) is the use of resistant cultivars. *C. canephora* is generally more resistant to leaf rust than *C. arabica* and thrives at higher temperatures (Harvey et al., 2021; Talhinhas et al., 2017), which can partially explain why Robusta coffee takes up such a large market share. However, this rust resistance is not absolute, and Robusta coffee is generally considered of lower quality and value (Harvey et al., 2021). Though switching to Robusta may reduce the price a farmer receives per kilogram of coffee, the higher and more reliable yield could provide more stable livelihoods to smallholder coffee farmers (Reay, 2019). Many coffee breeding programs are currently active worldwide, *e.g.*, in Brazil (Sera, 2001) and Ethiopia (Benti, 2017). To support these programs, knowledge about the underlying phylogeny of coffee and its crop wild relatives is essential. If the variation among species in certain desirable traits is better understood, breeding programs stand a better chance of successfully creating more optimized cultivars and variants. For long-term crop sustainability, sufficient genetic diversity of coffee crop wild relatives is necessary to constitute a viable gene pool for breeding purposes (Brozynska et al., 2016; Warschefsky et al., 2014). Wild *Coffea* species, such as *C. stenophylla*, have shown some promise as potential beverage species (Davis et al., 2021). Nonetheless, further research into the application of these genetic resources is still required (Brozynska et al., 2016; Warschefsky et al., 2014).

1.3 Research gap

The current research on plant functional traits is quite extensive, and continues to grow. A number of previous studies have compared functional traits in coffee (*e.g.*, Buchanan et al. (2019); Dubberstein et al. (2021); Dutra Giles et al. (2019)). However, these studies have remained limited to one or two species and have not included genus-wide species comparisons. Alternatively, there have been studies comparing traits between species and even between genera within a family (*e.g.*, Jordan et al. (2015); McCormack et al. (2020); Onstein et al. (2016)). Nonetheless, these comparisons have not been applied to the entire *Coffea* genus as of yet. Current knowledge on the evolutionary processes within the genus is incomplete. By further investigating the trait variability of coffee plants, we hope to gain a better understanding of the adaptive value of traits that have evolved in different *Coffea* species.

In this study, the aim is to estimate the distribution of intra- and interspecific variation in the *Coffea* genus for the following functional leaf traits: stomatal density, leaf area, SLA, leaf weight, and stomatal dimensions. We examine how these traits might have evolved across *Coffea* species, and we test for any potential relationships among leaf traits, or between leaf traits and climate. To what extent is there phylogenetic signal in the trait values, and how does this compare among traits? What might this tell us about the life history strategies of the different *Coffea* clades and species? Finally, in what way could this information be used to help promote more efficient and sustainable coffee cultivation across the globe?

In an attempt to answer these questions, we perform phylogenetic comparative analyses on leaf trait data. We investigate the evolution of functional leaf traits in the *Coffea* genus by analyzing the variance, estimating phylogenetic signal, and performing phylogenetic regressions between leaf traits and climatic variables. Additionally, we compare Brownian Motion, Ornstein-Uhlenbeck, and Early Burst models of evolution to investigate which processes might have marked the evolutionary history of *Coffea*.

We hypothesize that most leaf traits will exhibit more variation between species than within them. Since most *Coffea* species have a fairly narrow distribution and many species are endemic to small regions (Davis et al., 2006), we hypothesize that variation in climatic variables between species will be larger than within species. However, some species have wider ecological niches than others (Davis et al., 2006; Herrera and Lambot, 2017), and may therefore contain large amounts of intraspecific variation both in leaf traits and climatic variables. Certain leaf traits might be correlated with each other: for example, we expect stomatal density to be inversely related to stomatal size (Brodribb et al., 2013). We also expect a number of leaf traits to be correlated with climatic variables (*e.g.*, lower SLA in dry habitats), though these relationships will likely be relatively weak. Additionally, we expect different models of evolution to fit the data in different clades: for example, an EB model of evolution might be expected to be the best-fitting model in Madagascan species, due to evidence for adaptive radiation on the island (Anthony et al., 2010; Davis et al., 2006).

Chapter 2

Methods

2.1 Data acquisition

For all species included in this study, data on SLA, stomatal density, leaf dry area, and leaf dry weight were available from previous work on herbarium specimens at Meise Botanic Garden. Data on stomatal density had been gathered following the protocol described by Bauters et al. (2020). Microscopic images at 1000x magnification had been taken of three view fields per leaf, after which a stomatal detection model trained by deep learning was used to count the number of stomata in each view field. These values were converted to stomata per mm^2 to obtain the stomatal density values used in this study (For details on the stomatal detection model, see Bauters et al. (2020)).

These trait measurements were compiled for 780 leaves across 167 specimens of 62 species. The 62 included species are all *Coffea* species, except for four related outgroup species: three from the Coffeeae tribe (*Belonophora coriacea*, *Calycosiphonia spathicalyx*, and *Tricalysia congesta*) and one species from a different tribe within the Ixoroideae subfamily (*Bertiera iturensis*). Of the 58 *Coffea* species in the data set, two are former members of *Psilanthus*: *Coffea mannii* and *Coffea ebracteolata* (formerly *Psilanthus mannii* and *Psilanthus ebracteolatus*, which are the names that are used in this study for clarity). Five leaves were included per specimen, when available. If data for less than five leaves was available, all available leaves were included in the data set.

The existing data was supplemented with additional measurements of stomatal dimensions on the same accessions and leaves, when available. Stomatal measurements were performed on the 1000x magnified images provided by Meise Botanic Garden using ImageJ open access software version 1.53v (Schneider et al., 2012). In total, stomata of 743 leaves of 159 specimens were measured across 59 different species, including 55 *Coffea* species as well as the four related outgroup species in the Meise Botanic Garden data set. For each stoma, four characteristics were measured in accordance with the protocol applied by Savvides et al. (2012): stomatal length, stomatal width, pore length, and pore width (*i.e.*, aperture) (Figure 2.1). These measurements were performed on three stomata per leaf and repeated for five leaves per specimen, unless no five leaves were available. Stomata were randomly selected from different sections of the leaf to avoid obtaining biased measurements. The three stomatal measurements per leaf were averaged to obtain unique trait values for each leaf. In total, 2229

stomata were measured across 743 leaves. All measured leaves were obtained from the same unique specimens and species as the data provided by Meise Botanic Garden. This allowed for the merging of the stomatal measurements with the Meise Botanic Garden data, resulting in a data set with trait data for 780 leaves (Supplementary material S1). Additionally, trait values were averaged for each species to obtain a separate data set with unique trait values for each species (Supplementary material S2). Due to a lack of source material, stomatal dimensions were not measured for three species: *C. homollei*, *C. arenesiana*, and *C. coursiana*.

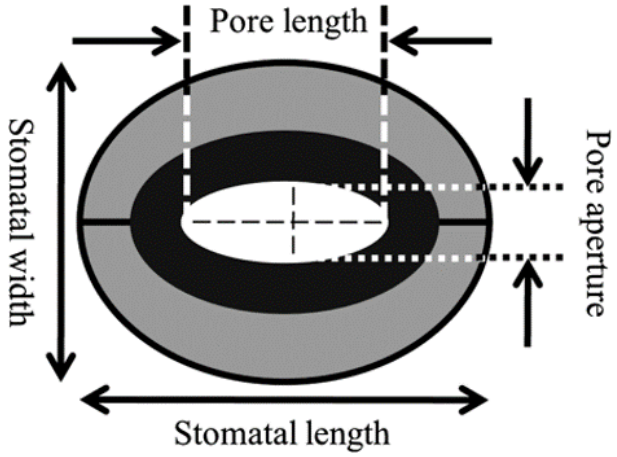


Figure 2.1: Stomatal proportions measured for each stoma. Adopted from Savvides et al. (2012).

Meise Botanic Garden provided geographic coordinates of georeferenced samples, as well as average altitudes, bioclimatic variables, and a discrete "dryness" trait value representing dry, humid, or seasonal habitat for each species. Dryness categories for the four outgroup species had not been previously assigned and were roughly determined on the basis of a Principal Component (PC) axis representing (lack of) precipitation (see 3.4, PC2). Species were assigned to the "Dry" category if $PC2 > 1$, the "Seasonal" category if $0 < PC2 < 1$, and the "Humid" category if $PC2 < 0$ (see Figure D.1 in Appendix D). Species were also assigned a "Region" category, based on occurrence in continental Africa or the West Indian Ocean Islands (occasionally simply denoted "Madagascar", though note that some samples were also included from Comoros and Mayotte (*C. humblotiana*), Réunion (*C. mauritiana*), and Mauritius (*C. macrocarpa*) (Figure 2.2).

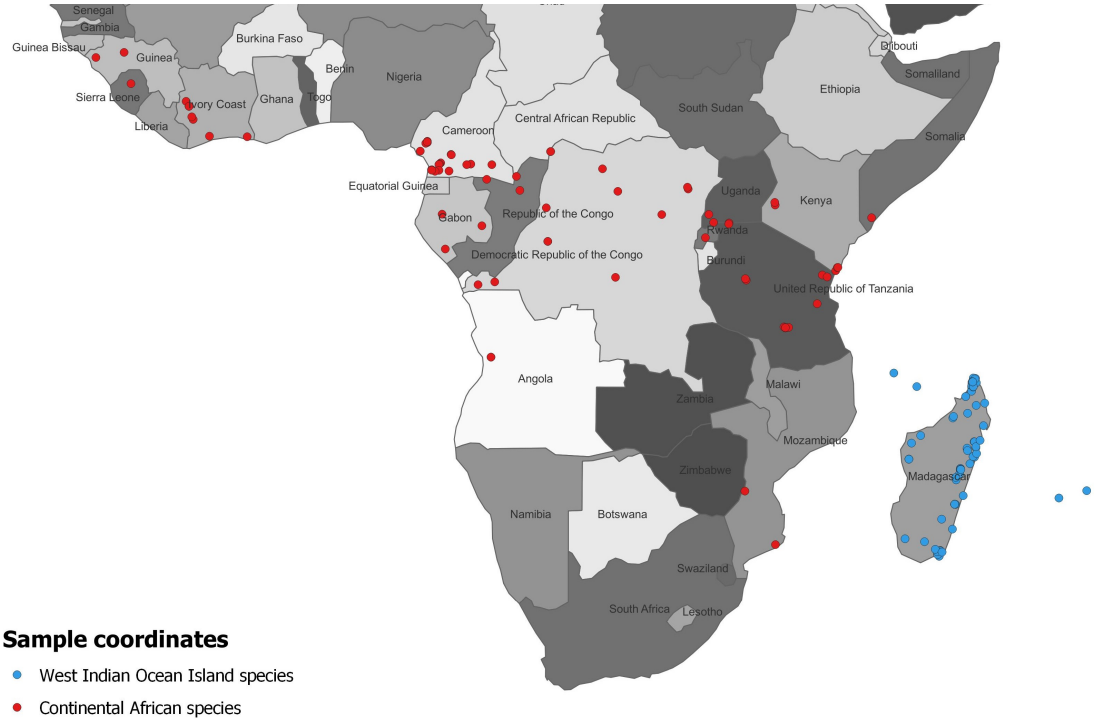


Figure 2.2: Map depicting sampling locations for all included specimens, colored according to occurrence in continental Africa (AFR) or the West Indian Ocean Islands (WIOI).

Shrinkage correction

Leaves can shrink considerably in size when dried, which can lead to biased inferences when working with herbarium accessions (Blonder et al., 2012). To account for this bias, shrinkage was measured for all available species in the living collection of Meise Botanic Garden greenhouses. Five healthy, adult leaves per plant were sampled for up to five accessions per species. The leaves were sampled randomly from each plant, excluding the first two to three juvenile leaves from the end meristem of each branch. If less than five accessions were available for a species, all viable accessions were sampled. In total, 145 leaves were sampled from 29 accessions, belonging to 11 different species (plus three varieties: *C. arabica* var. *bourbon*, *C. arabica* var. *laurina*, and *C. liberica* var. *liberica*). A full list of the sampled specimens is available in Supplementary material S3. The fresh leaves were weighed using a precision scale (1 mg accuracy), and scanned using a flatbed scanner (Canon CanoScan 9000F Mark II). Petioles were removed before scanning to ensure standardized area measurements across samples. Leaves were then dried in a herbarium oven until fully dry. The dry leaves were again weighed and scanned using the same equipment. Fresh and dry leaf area were measured with ImageJ open access software version 1.53v (Schneider et al., 2012). For each leaf, a shrinkage factor was calculated as the ratio of dry area to fresh area. Since Blonder et al. (2012) found leaf dry area to be the best predictor of leaf shrinkage across species, we used dry area data to estimate shrinkage for each herbarium leaf. A simple linear regression found the natural logarithm of leaf dry area to be a significant predictor of shrinkage factor ($Shrinkage\ factor = 0.008851 * log_e(Dry\ area) + 0.895520$, $P < 0.001$, though residuals deviated from normality: Shapiro-Wilk normality test, $W = 0.87496, P < 0.001$). Data on SLA was divided by the obtained shrinkage factors, resulting in SLA data with a correction for leaf shrinkage.

2.2 Data analysis

All analyses were performed in R version 4.2.3 (R Core Team, 2022). The phylogeny used for all phylogenetic analyses was based on Hamon et al. (2017). It is important to note, however, that this tree is merely the most likely hypothesis for the phylogenetic relationships of the studied species. Although the assumed taxonomic structure is highly supported, some caution should still be exercised when making inferences based on it. Hamon et al. (2017)'s summarized phylogenetic tree was pruned using the `drop.tip` function in the R package `APE` (Paradis and Schliep, 2019) in order to retain only species that were included in our data set (Figure B.1 in Appendix B). Additionally, some of our analyses require a fully ultrametric tree (*i.e.*, a tree with all tips being equidistant from the root). We used the `chronos` function in R package `APE` to ensure ultrametricity. The height of the full tree, including outgroups, was set to 1. After checking that the full phylogeny was ultrametric and dichotomous, a number of subclades of the full phylogeny were isolated from the full phylogenetic tree using the `drop.tip` and `extract.clade` functions from `APE`. Subtrees were created for the full *Coffea* genus (*i.e.*, omitting outgroup species), the Eu-coffee (EC) clade, the African (AFR) subclade, and the West Indian Ocean Islands (WIOI) subclade. The full tree including outgroups was scaled to length 1, and relative lengths of the subtrees were recorded for later reference. Additionally, we generated distinct data sets at the same taxonomic levels as the subtrees. These subtrees allowed us to perform independent analyses at different taxonomic levels within our data, and to compare results between African and Madagascan species. Because our data set only included three species in the Xeno-coffee (XC) clade, we did not perform independent analyses on this clade.

Before analyzing the data, correlations between the measured traits were tested to avoid multicollinearity or redundancy in the leaf data. The Spearman correlation coefficient (ρ) was calculated for all pairwise trait combinations (Table 2.1). If ρ exceeded 0.8, one of the correlated traits was dropped from the data set. We found leaf weight to be highly correlated with leaf area ($\rho = 0.898, P < 0.001$), and pore length was highly correlated with stomatal length ($\rho = 0.817, P < 0.001$). Consequently, leaf weight and pore length were no longer considered in further analyses. The correlation between stomatal length and stomatal width also approached the threshold for removal ($\rho = 0.794, P < 0.001$). However, we deemed this trait of sufficient interest to be regarded as a separate variable. Our final analyses included six leaf traits: stomatal density (expressed in stomata per mm^2), leaf area (expressed in m^2), SLA (expressed in m^2/kg), stomatal length, stomatal width, and pore width (all expressed in µm). Leaf area was natural log-transformed (further simply denoted as log(leaf area)) to avoid violating the assumption of normally distributed residuals in further analyses. Finally, an outlying observation was removed from the leaf data set (*C. stenophylla*, specimen Vermoesen 2182; see Supplementary material S1). This leaf had a distinctly lower weight than the other leaves from the same sample, despite being around five times larger in surface area. Species average trait values for *C. stenophylla* were recalculated after removing this outlier. This reduced the number of leaves in the leaf data set to 742. For the species data set, Standard Error (SE) was also calculated for each leaf trait based on the individual leaf data.

Table 2.1: Matrix of Spearman correlation coefficients for all pairwise combinations of measured leaf traits.

Trait	Leaf Area	Leaf Weight	Stom. Density	SLA	Stom. length	Stom. width	Pore length	Pore width
Leaf Area	*1.00*							
Leaf Weight	**0.90**	*1.00*						
Stomatal Density	-0.11	0.07	*1.00*					
SLA	0.16	-0.24	-0.44	*1.00*				
Stomatal length	0.24	0.11	-0.51	0.25	*1.00*			
Stomatal width	0.23	0.13	-0.35	0.17	0.79	*1.00*		
Pore length	0.19	0.05	-0.55	0.28	**0.82**	0.62	*1.00*	
Pore width	0.03	-0.01	-0.30	0.07	0.49	0.49	0.51	*1.00*

Correlations where $\rho > 0.8$ are marked in **bold**.

Intra- and interspecific variance

In order to obtain a general overview of the distribution of the variance in trait values, the proportion of the variance explained by between-species differences was estimated for each trait using a one-way ANOVA. Additionally, trait value ranges for each trait were plotted in box plots per species to visualize the within and between species variance in the data.

A PCA was performed to visualize the axes of trait variation in the data set. However, PCA assumes that the data points are independent (Revell, 2009). Because of the phylogenetically correlated nature of the data, a standard PCA would be statistically inappropriate. The R package `PHYTOOLS` (Revell, 2012b) contains the function `phyl.pca`, which accounts for phylogenetic relatedness in a PCA. Each Principal Component (PC) then represents an axis of multivariate trait evolution, decreasing in magnitude from PC1 onward. We used this function to decompose our leaf trait data into phylogenetically structured PCs. The phylogenetic PCA was performed on the correlation matrix in stead of the covariance matrix, due the fact that the traits were expressed in different units of measurement. We plotted the results in a traditional biplot, as well as using the `PHYTOOLS` function `phylomorphospace` to visualize the phylogenetic relationships of the data points and their position along the first two PC axes.

Phylogenetic signal

Phylogenetic signal in univariate trait values was estimated using the `phylosig` command in the R package `PHYTOOLS`. We used Pagel's lambda (λ) (Pagel, 1999) as a measure of phylogenetic signal, while accounting for the standard error of the data. Signal was evaluated separately for each trait and in each clade, providing an insight in the degree of phylogenetic clustering of the individual traits. However, these estimates do not take into account the effect

of other variables on signal in any given trait. Therefore, we also estimated phylogenetic signal simultaneously with the phylogenetic regressions later on in the analyses (see '2.2: Phylogenetic regressions' for details). To visualize the evolution of the traits across the phylogeny, trait values were mapped onto the phylogeny using the PHYTOOLS function contMap. Outgroups were included in the trait-to-tree maps, since some traits only displayed significant phylogenetic signal when outgroups were included.

Models of continuous trait evolution

To investigate which processes might have impacted the evolution of the species in our phylogeny, we fitted three different models of continuous trait evolution to our trait data. Specifically, we assessed the likelihood of BM, OU, and EB models of evolution for each trait in our data set with fitContinuous in the R package GEIGER (Pennell et al., 2014), accounting for measurement error in our trait data. Models were run for 500 iterations. We used Akaike Information Criterion (AIC) values to determine which of the fitted models best described our data for each trait, with lower AIC values indicating a better fit. The fit of the three tested models was compared across four different clades to be able to draw more specific conclusions about the evolution of the studied traits. The compared clades were the complete *Coffea* genus, the EC clade, the AFR subclade, and the WIOI subclade. AIC values were visualized in bar plots for the different models to determine the relative fit of the models to the data.

To corroborate the parameter estimates of the best-fitting models in the full *Coffea* genus obtained using fitContinuous, the package OUWIE (Beaulieu and O'Meara, 2022) was used to fit the same models. The best-fitting model for each trait was fit to the data with OUwie as an OU1 model, *i.e.*, an OU model with a single optimum, also including standard errors in the model. The parameters obtained from the OUwie model deviated from those obtained by fitContinuous, but remained in the same order of magnitude. The parameters were deemed sufficiently similar to the ones obtained previously in order to rule out potential model specification errors.

To further investigate the evolutionary patterns in the trait data, we used OUwie to fit OU models with multiple optimal trait values (OUM models) in the *Coffea* genus, not including outgroup species. In these models, we allowed species from dry, humid, and seasonal climates to have different optima based on the value of their categorical "Dryness" variable. We compared the AIC values for these OUM models to those obtained in the single optimum OU models to assess their relative fit. To minimize errors, the AIC values of the OU1 models from OUwie were used for model comparison in stead of those obtained from fitContinuous in package GEIGER.

Phylogenetic regressions

Six multiple phylogenetic Generalized Least Squares (pGLS) regressions were performed to test for relationships between traits and climate or environmental variables. Outgroups were not included in the regressions. Each time, a different leaf trait was taken as the dependent variable. The tested independent variables included the five other leaf traits, dryness (a categorical variable with three levels: dry, seasonal or humid), altitude, latitude, longitude, and 19 bioclimatic variables drawn from WorldClim (Fick and Hijmans, 2017) for the given coordinates. To reduce the dimensionality of the bioclimatic variables, a PCA was applied to

the climate data. PCs were retained until the individual component explained less than 10% of the total variance in the data and the PC was still straightforward to interpret biologically. The retained PCs were added to the data set and used as independent variables in the phylogenetic regression analyses in stead of the 19 bioclimatic variables. The abbreviations used for the original bioclimatic variables are explained in Table 2.2.

Table 2.2: Abbreviations used for the 19 bioclimatic variables drawn from Fick and Hijmans (2017), and their definitions.

Abbreviation	**Definition**
AMTEMP	Annual Mean Temperature
MDUR	Mean Diurnal Temperature Range (Mean of monthly difference between maximum and minimum temperature)
ISOTHER	Isothermality (MDUR/TEMPAR ∗100)
TEMPSEAS	Temperature Seasonality (Standard deviation ∗100)
MAXTWM	Maximum Temperature of the Warmest Month
MINTCM	Minimum Temperature of the Coldest Month
TEMPAR	Temperature Annual Range (MAXTWM - MINTCM)
MTWETQ	Mean Temperature of Wettest Quarter
MTDRYQ	Mean Temperature of Driest Quarter
MTWARMQ	Mean Temperature of Warmest Quarter
MTCOLDQ	Mean Temperature of Coldest Quarter
APREC	Annual Precipitation
PWETM	Precipitation of Wettest Month
PDRYM	Precipitation of Driest Month
PSEAS	Precipitation Seasonality (Coefficient of variation)
PWETQ	Precipitation of Wettest Quarter
PDRYQ	Precipitation of Driest Quarter
PWARMQ	Precipitation of Warmest Quarter
PCOLDQ	Precipitation of Coldest Quarter

The decision whether to apply a phylogenetic correction to a linear regression depends on the presence and strength of phylogenetic signal in the data. However, it has been shown that basing this decision solely on estimates of phylogenetic signal in individual traits can lead to erroneous inferences (Hansen and Orzack, 2005; Revell, 2010). To correctly control for phylogenetic relatedness in regressions, it is necessary to estimate the quantifier of phylogenetic signal (Pagel's λ in this study) simultaneously with the regression model (Hansen and Orzack, 2005; Vandelook et al., 2012). We therefore implemented our regressions using a Maximum Likelihood (ML) approach that simultaneously estimates λ with the R package `CAPER` (Orme et al., 2018). This method allows us to account for the effects of other variables on the trait while estimating its phylogenetic signal, and simultaneously accounting for this signal in the regression analysis. For each pGLS regression model, we used a backward selection approach to remove nonsignificant predictors one by one, until the AIC value reached a minimum.

Chapter 3

Results

3.1 Intra- and interspecific variance

The distribution of the variance of the six measured traits in the *Coffea* genus is shown in Figure 3.1 (A-F). For all traits, significant differences were detected between species ($P < 0.0001$ in all cases). Between-species differences explained the majority of the variance in all traits, except pore width (Stomatal density: 63%, leaf area: 73%, SLA: 76%, stomatal length: 64%, stomatal width: 57%, pore width: 41%). Though some species exhibited wider trait value ranges than others, differences between species were generally greater than within-species variation. Differences between species were the greatest in SLA and leaf area; both of these traits showed relatively little variation within species (Figure 3.1 B, C). In contrast, most of the variation in pore width (59%) was intraspecific (Figure 3.1 F).

The phylogenetic PCA of the trait data resulted in the PC axes shown in figures 3.2 and 3.3 (A-C). The loadings, eigenvalues and (cumulative) variance explained by the PCs are given in Table 3.1. The first two PCs jointly explained 64% of the variance in the data (PC1: 42%, PC2: 22%). PC1 had strongly positive loadings for stomatal length (+0.94), stomatal width (+0.86), and pore width (+0.70), as well as a negative loading for stomatal density (-0.57). This axis thus generally represents a continuum from species with small stomata, to species with large stomata. Species with smaller stomata also tend to have higher stomatal density according to this PC axis. PC2 is somewhat harder to interpret, with a relatively strong negative loading for SLA (-0.66) and positive loadings for stomatal density and leaf area (both +0.63). We therefore take the PC2 axis to represent an axis of leaf investment, ranging from small leaves with lower stomatal density and higher SLA, to larger leaves with more stomata per mm^2 and higher SLA.

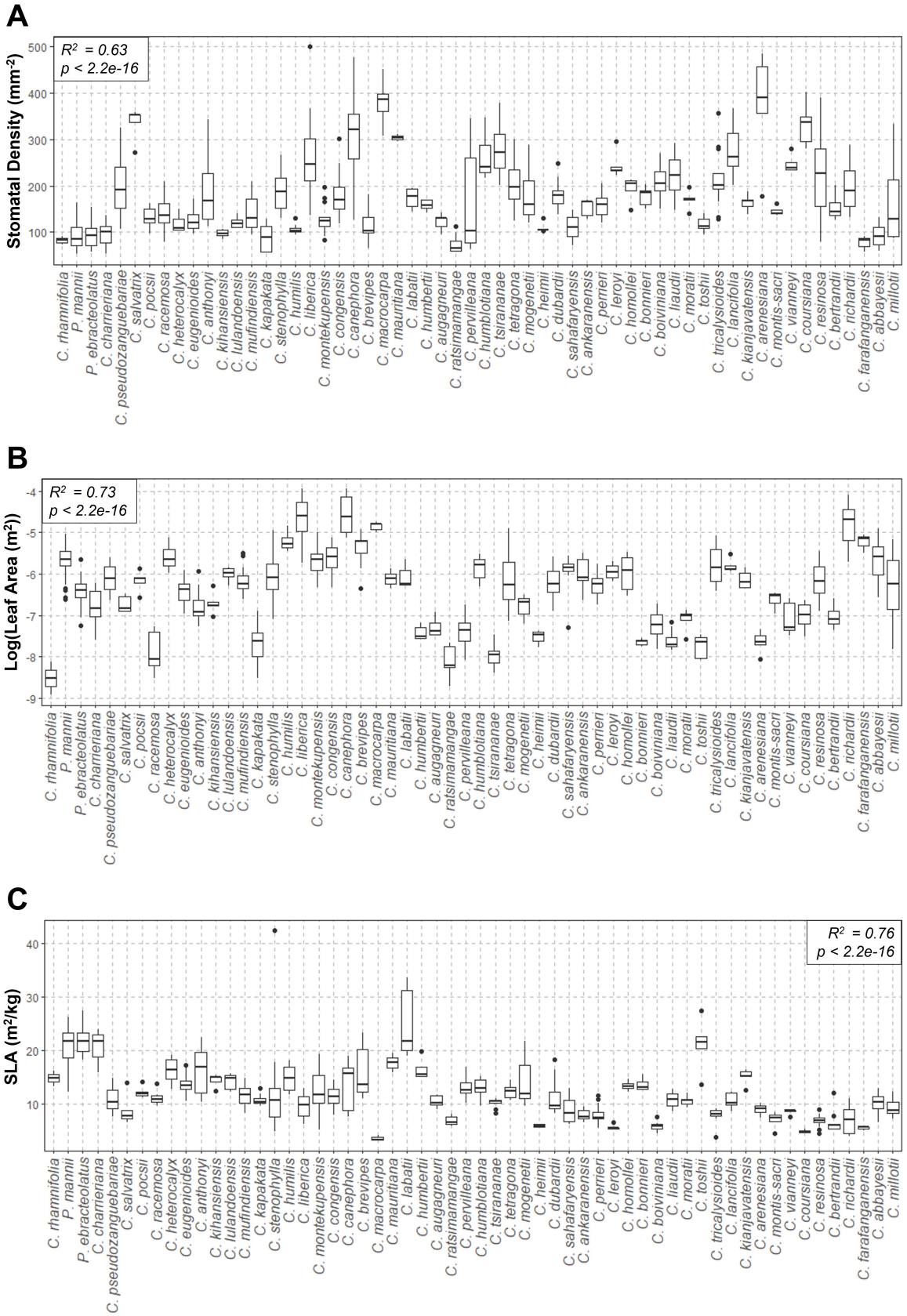
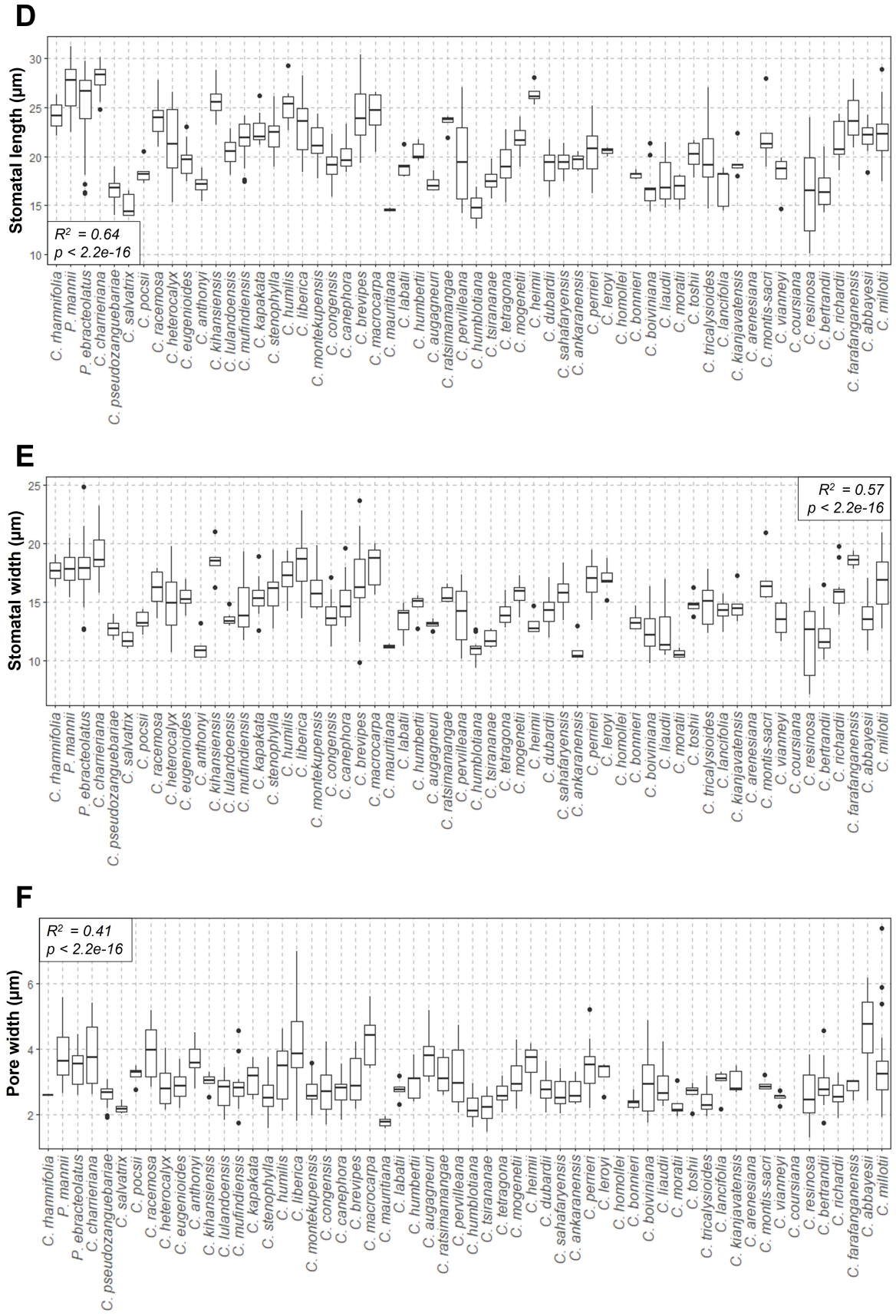


Figure 3.1: Box plots showing variation in each of the six measured leaf traits. R^2 and p-values are given in each figure. Outgroup species were not included in the plots, nor in the calculation of the shown statistics. (Continued on next page)



(Continued) Box plots showing variation in each of the six measured leaf traits. R^2 and P values are given in each figure. Outgroup species were not included in the plots, nor in the calculation of the shown statistics.

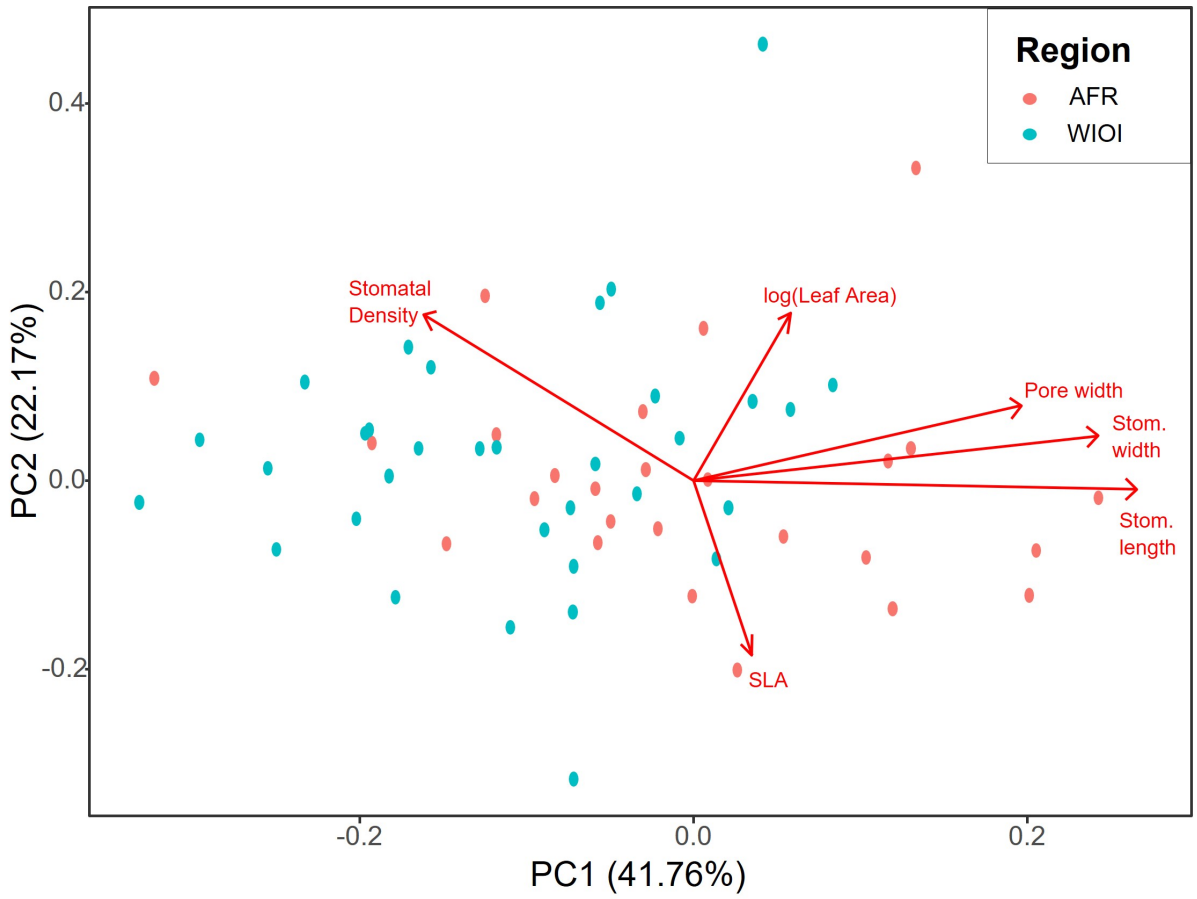


Figure 3.2: Biplot showing the first two PC axes of the trait phylogenetic PCA. Arrows indicate loadings; points indicate species. Species are colored based on occurrence in continental Africa (AFR) or the West Indian Ocean Islands (WIOI).

Table 3.1: Loadings for the first two PC axes of the leaf trait phylogenetic PCA. Eigenvalues and (cumulative) proportions of variance explained by the PCs are also given.

Trait	**PC1**	**PC2**
Stomatal density	-0.57	0.63
log(Leaf area)	0.21	0.63
SLA	0.12	-0.66
Stomatal length	0.94	-0.03
Stomatal width	0.86	0.17
Pore width	0.70	0.28
Variance explained	0.42	0.22
Cumulative variance	0.42	0.64
Eigenvalue	2.51	1.33

Species occupying the more basal branches of the phylogeny, such as the outgroups, the XC clade, and *C. charrieriana*, tend to have positive values for PC1 (Figure 3.3A), indicating that these lineages have relatively large stomata and lower stomatal density compared to more derived species in the AFR or WIOI subclades. Excluding the outgroups, the more basal *Coffea* species also display lower PC2 scores than younger species. Furthermore, African species seem to have higher PC1 values than Madagascan species (Figure 3.3B). Habitat dryness is relatively evenly distributed across PC1, but shows a clear distinction along PC2 (Figure 3.3C). Species in humid environments tend to have higher PC2 scores than species with seasonal or dry climates. Species in humid environments therefore generally have larger leaves with higher stomatal density and lower SLA. Notably, two closely related species, *C. mauritiana* and *C. macrocarpa*, have highly divergent traits: *C. mauritiana* has very low PC1 values and is intermediate on the PC2 axis, whereas *C. macrocarpa* has high values for both PC axes.

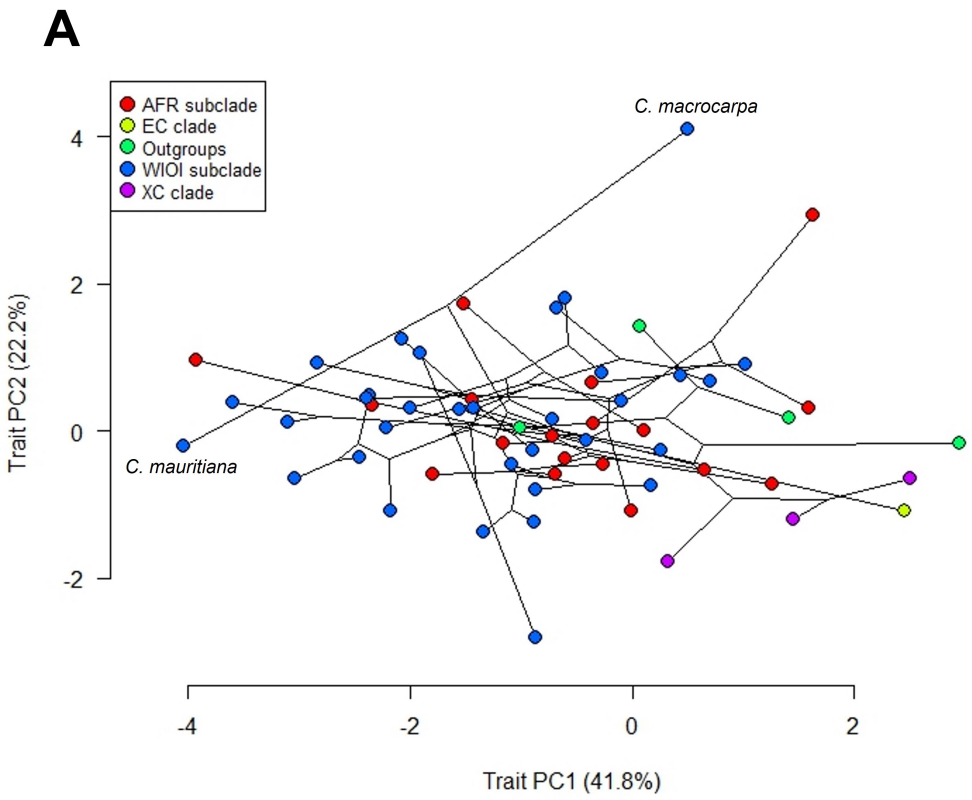
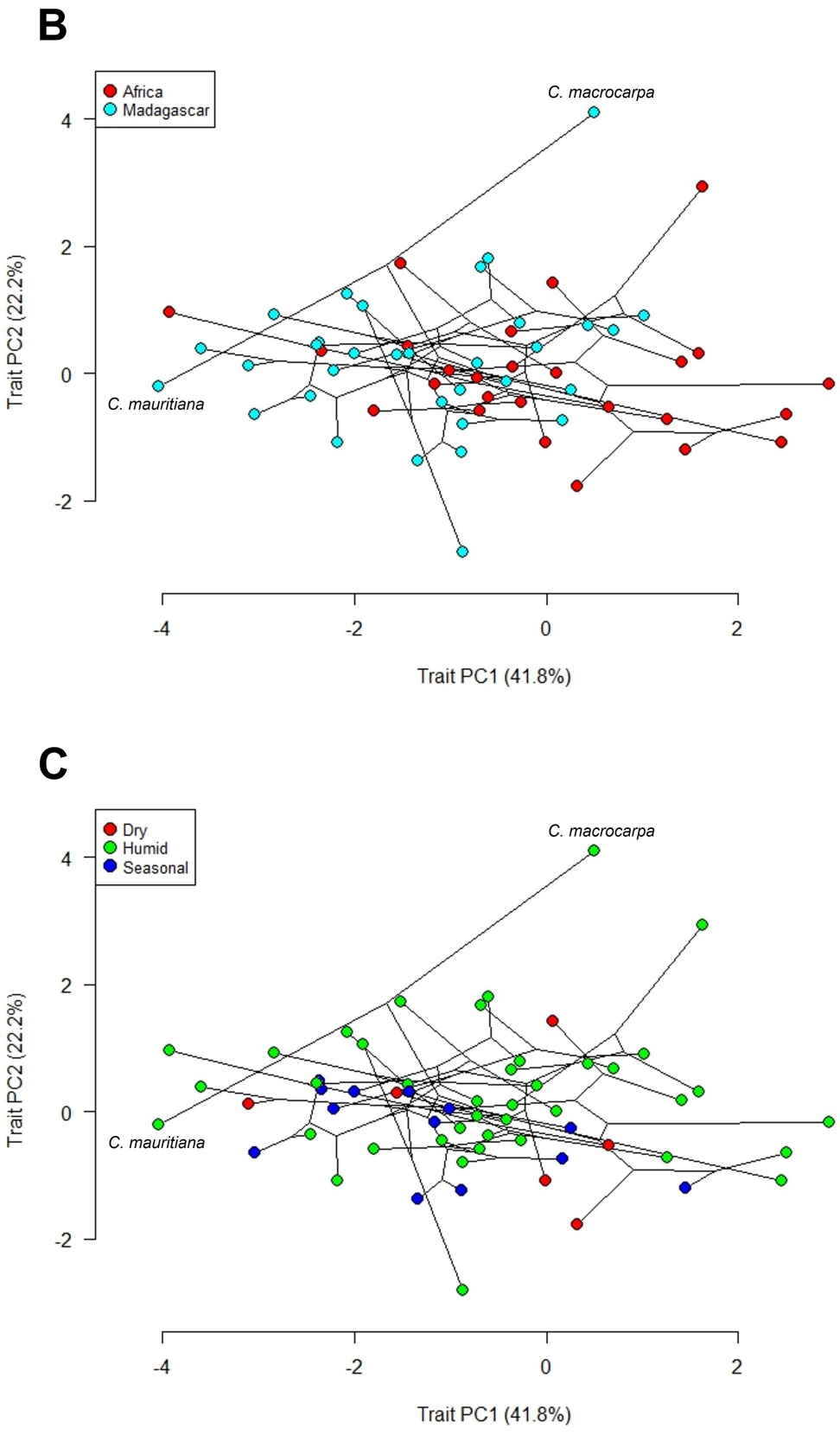


Figure 3.3: Phylomorphospace of the trait data across all species, including outgroups. The first two PCs are shown. Colors indicate **A**: clade, **B**: region of occurrence, **C**: dryness category. Lines indicate phylogenetic relatedness. (Continued on next page)



(Continued) Phylomorphospace of the trait data across all species, including outgroups. The first two PCs are shown. Colors indicate **A**: clade, **B**: region of occurrence, **C**: dryness category. Lines indicate phylogenetic relatedness.

3.2 Phylogenetic signal

Trait variation and univariate phylogenetic signal across the phylogeny is visualized in Figure 3.4 (A-D). In the subclades of the *Coffea* genus, none of the tested traits showed significant phylogenetic signal (*i.e.*, λ significantly different from 0), except for stomatal density in the WIOI subclade (Table 3.2). When considering the full genus, phylogenetic signal became significant in SLA ($\lambda = 0.52, P < 0.01$) and in stomatal length ($\lambda = 0.73, P < 0.01$). When including outgroups in the considered tree, stomatal length ($\lambda = 0.75, P < 0.01$) and stomatal width ($\lambda = 0.57, P < 0.01$) displayed significant signal, whereas phylogenetic signal in SLA became slightly less significant ($\lambda = 0.54, P < 0.05$). All other traits were randomly distributed across the phylogeny, with more related species not being significantly more similar to each other than less related lineages.

Table 3.2: Phylogenetic signal estimated univariately for each trait across different clades in the phylogenetic tree.

Clade	With outgroups		*Coffea*		EC		AFR		WIOI	
	λ	P	λ	P	λ	P	λ	P	λ	P
Stomatal density	0	n.s.	0	n.s.	0	n.s.	0	n.s.	**0.69**	*
log(Leaf area)	0.68	n.s.	0.69	.	0.55	.	0.62	n.s.	0	n.s.
SLA	**0.54**	*	**0.52**	**	0.27	n.s.	0.43	n.s.	0	n.s.
Stomatal length	**0.75**	**	**0.73**	**	0.70	n.s.	0.83	n.s.	0	n.s.
Stomatal width	**0.57**	**	0.36	n.s.	0	n.s.	0.26	n.s.	0	n.s.
Pore width	0	n.s.	0	n.s.	0	n.s.	0	n.s.	0	n.s.

Significance: n.s., $P > 0.10$; . , $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$.

The stomatal density of the species in the WIOI subclade ranged from high values in the subclade's basal lineages (*C. macrocarpa*, *C. mauritiana*) to lower values in some more recently derived species (*e.g.*, *C. ratismamangae*, *C. farafanganensis*) (Figure 3.4A). However, some more recent species from within the subclade also had higher stomatal densities (*e.g.*, *C. arenesiana*). SLA values were intermediate for the outgroups, with generally higher values basally in the *Coffea* genus (*i.e.*, the XC clade, as well as *C. charrieriana*), and lower values in more recent species of the WIOI subclade (Figure 3.4B). Stomatal length and stomatal width showed similar patterns across the phylogeny, with high values in the outgroups (Figure 3.4C, D). Stomatal length was particularly high in the basal lineages, whereas these species had relatively high, but less extreme stomatal width values.

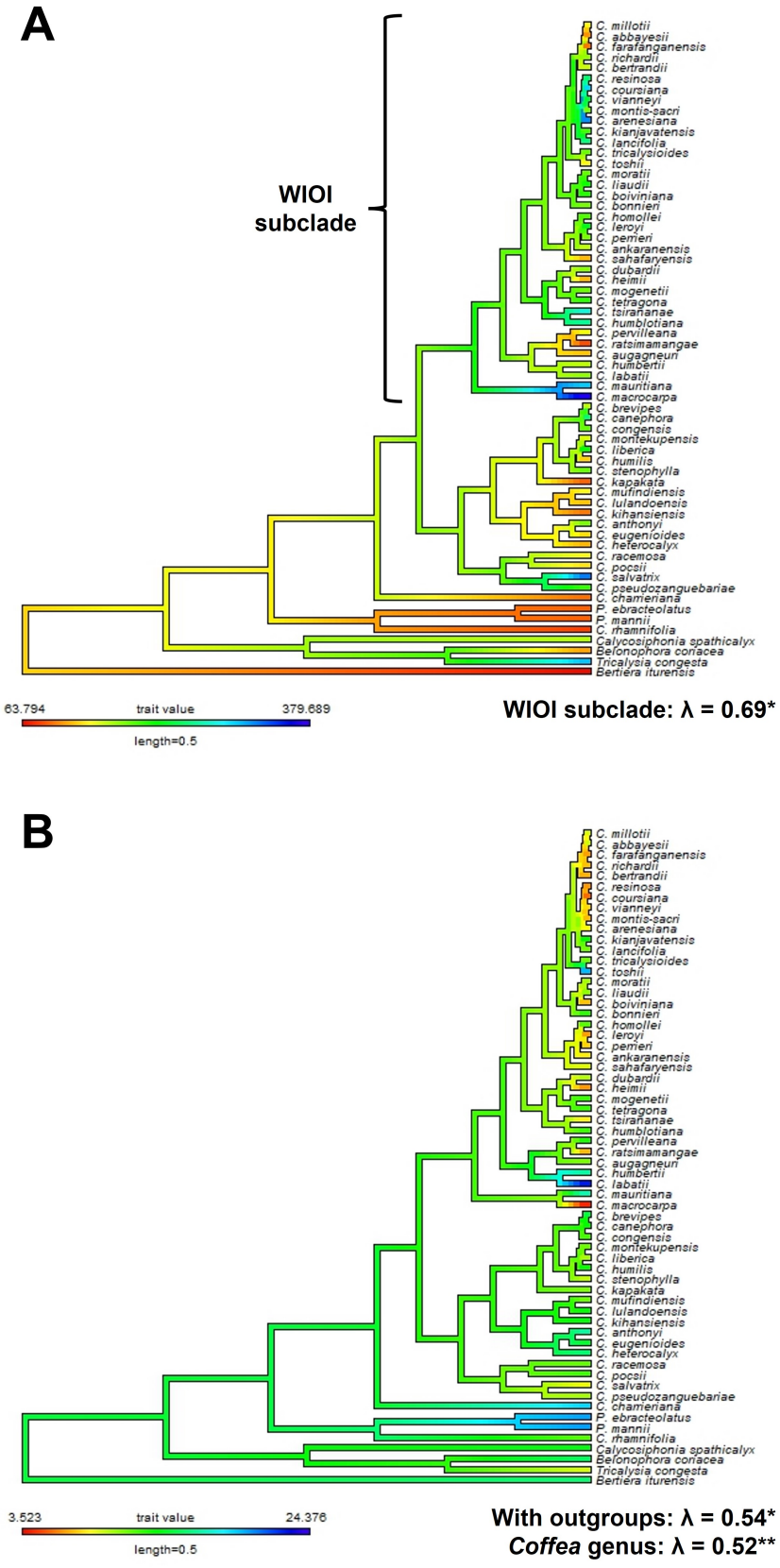
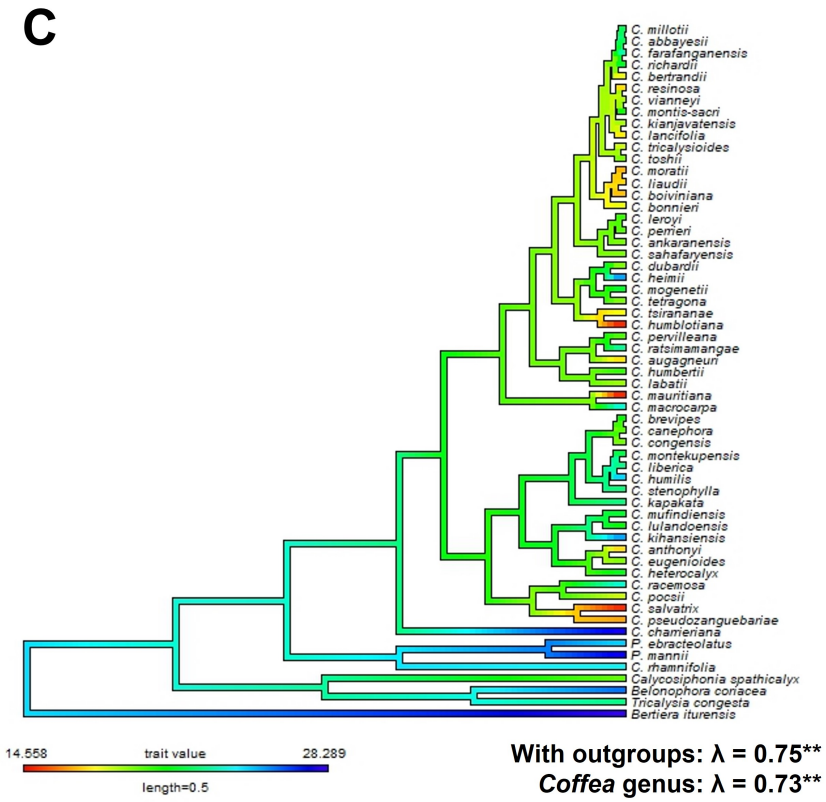


Figure 3.4: Trait-to-tree maps showing trait levels across the phylogeny. Only traits with significant phylogenetic signal are shown. **A**: Stomatal density; **B**: SLA; **C**: Stomatal length; **D**: Stomatal width. *, $P < 0.05$; **, $P < 0.01$. (Continued on next page)



(Continued) Trait-to-tree maps showing trait levels across the phylogeny. Only traits with significant phylogenetic signal are shown. **A**: Stomatal density; **B**: SLA; **C**: Stomatal length; **D**: Stomatal width. *, $P < 0.05$; **, $P < 0.01$.

3.3 Models of continuous trait evolution

The AIC values of all fitted models are shown in Figure 3.5. Parameter estimates for the best-fitting model for each trait in each clade are given in Table 3.3.

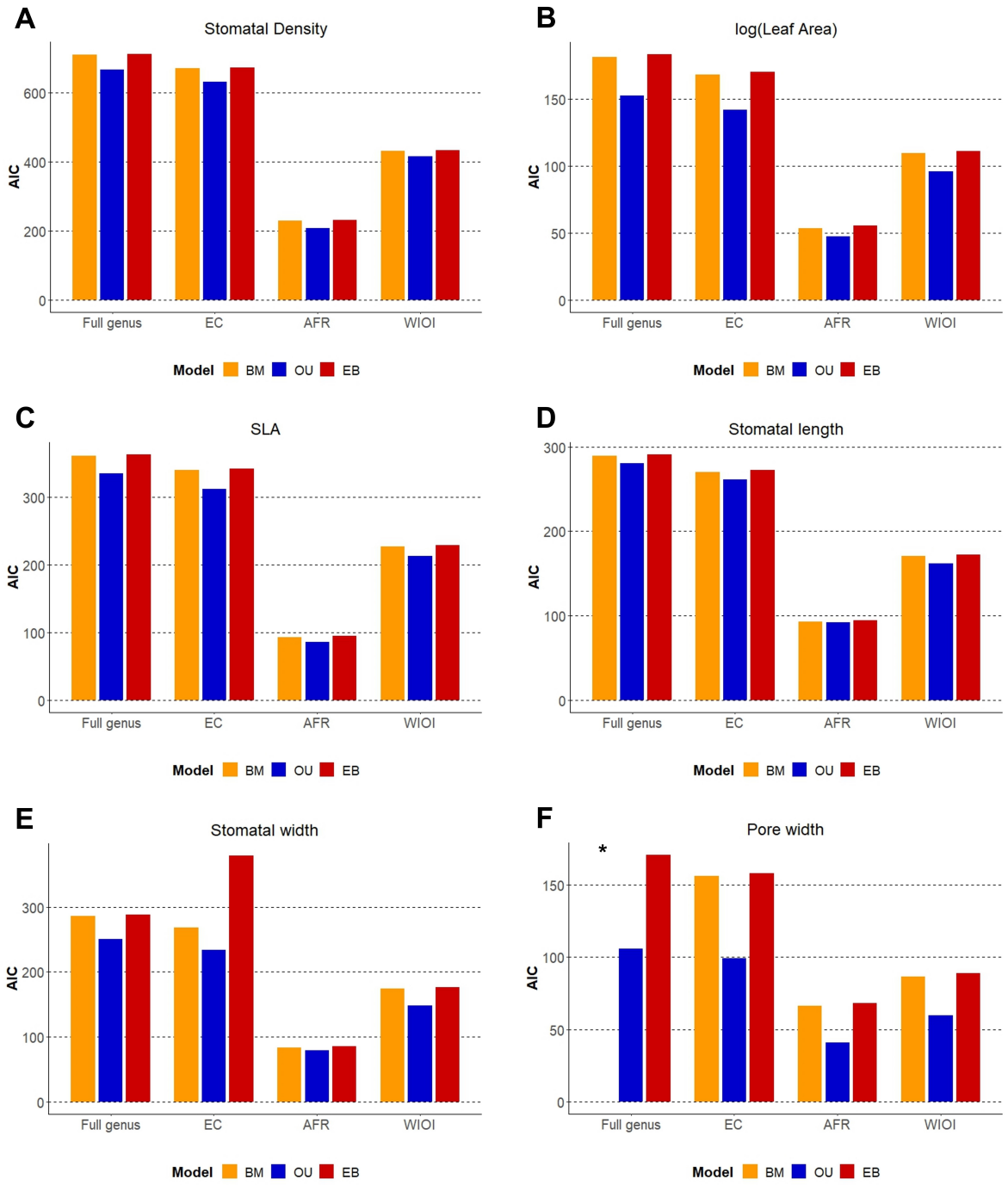


Figure 3.5: AIC values for the tested models of trait evolution. X-axes show the considered clade. Bar color corresponds to the three tested models. The lowest AIC value indicates the best fitting model for each clade-trait combination. The asterisk (*) in panel **F** indicates an extremely high AIC value (2890), which was not plotted to avoid distorting the rest of the figure.

Table 3.3: Parameter values for the best-fitting model for each trait across all considered clades, not including outgroups.

Clade	Trait	Best-fit model	Parameters				AIC
			σ^2	θ	α	$t_{1/2}$	
Coffea (0.563)	Stom. density	OU	2.65E6	170.47	304.16	0.40%	666.28
	log(Leaf area)	OU	53.67	-6.48	33.59	3.66%	152.42
	SLA	OU	1597.56	12.01	47.32	2.60%	334.94
	Stom. length	OU	263.16	21.22	10.21	12.07%	280.69
	Stom. width	OU	1010.83	14.55	105.54	1.17%	250.48
	Pore width	OU	211.51	3.07	307.66	0.40%	105.65
EC (0.375)	Stom. density	OU	2.58E6	175.35	305.68	0.61%	630.75
	log(Leaf area)	OU	53.30	-6.44	36.12	5.12%	141.58
	SLA	OU	1752.89	11.45	61.68	2.99%	311.99
	Stom. length	OU	344.58	20.28	17.37	10.64%	260.92
	Stom. width	OU	954.48	14.35	107.56	1.72%	233.34
	Pore width	OU	205.09	3.05	302.47	0.61%	99.39
AFR (0.225)	Stom. density	OU	2.71E7	161.22	3402.21	0.09%	207.62
	log(Leaf area)	OU	30.43	-6.27	24.20	12.71%	47.40
	SLA	OU	1499.53	12.36	172.32	1.79%	85.91
	Stom. length	OU	189.81	20.43	10.28	29.96%	92.08
	Stom. width	OU	153.49	14.60	21.49	14.36%	78.88
	Pore width	OU	6317.45	3.17	7995.23	0.04%	40.89
WIOI (0.200)	Stom. density	OU	1.38E6	186.79	166.66	2.08%	415.35
	log(Leaf area)	OU	81.58	-6.59	56.52	6.10%	95.83
	SLA	OU	2468.22	10.53	74.55	4.65%	213.35
	Stom. length	OU	512.40	19.37	36.95	9.40%	161.62
	Stom. width	OU	1234.17	13.95	152.17	2.28%	147.63
	Pore width	OU	144.25	2.95	252.57	1.37%	59.68

OU = Ornstein-Uhlenbeck; σ^2 = Brownian rate of evolution; θ = optimal trait value; α = strength of attraction towards θ; $t_{1/2}$ = phylogenetic half-life ($log_e(2)/\alpha$), expressed as a percentage of the considered phylogeny; AIC = Akaike Information Criterion. Values between brackets indicate the root-to-tip length of each tree, relative to the full tree with outgroups (which was scaled to length 1).

For all tested traits in each clade, the OU model significantly outperformed the BM and EB models. Only for stomatal length and stomatal width in the AFR subclade, AIC values for BM and EB models were comparable to that of the OU model. For stomatal length in the AFR subclade, the relative likelihood (AIC weight) for the OU model was 0.51, with BM and EB models obtaining values of 0.36 and 0.13, respectively. Relative likelihoods for stomatal width in the AFR subclade were 0.84 for OU, 0.12 for BM, and 0.04 for EB. In all other cases, the relative likelihood of the OU model exceeded 0.90, usually approaching 1.

The multiple optimum (OUM) models outperformed the single optimum (OU1) models for two traits: leaf area and stomatal width (Table 3.4). OU1 models performed slightly better for the other four considered leaf traits. Model parameters estimated with `OUwie` were generally of similar magnitude to those of the `fitContinuous` models (tables 3.3, 3.4). In the OUM models, the trait optimum for log(leaf area) was smallest in dry habitats (θ_D = -8.01) and largest in humid habitats (θ_D = -6.16), with an intermediate optimum for seasonal environments (θ_S = -6.72). Stomatal width exhibited the largest optimum in humid areas (θ_H = 14.96), with a slightly lower optimum in dry habitats (θ_D = 14.80) and the lowest optimal trait value in environments with seasonal dryness (θ_S = 13.37).

Table 3.4: Parameter values for the best-fitting OU model from R package OUwie. Models were fit across the full *Coffea* genus, and outgroups were not included.

Parameter	**Stom. Density**	**log(Leaf area)**	**SLA**	**Stom. length**	**Stom. width**	**Pore width**
σ^2	$2.13*10^6$	32.45	845.29	148.05	2511.64	118.97
α	246.25	28.59	24.39	5.74	292.11	173.06
$t_{1/2}$	0.50%	4.31%	5.05%	21.47%	0.42%	0.71%
θ	169.03		12.01	21.22		3.07
θ_D		-8.01			14.80	
θ_H		-6.16			14.96	
θ_S		-6.72			13.37	
OUM AIC	628.21	**133.35**	321.79	282.28	**249.90**	109.07
OU1 AIC	**625.80**	143.12	**318.14**	**280.69**	250.48	**105.65**

σ^2 = Brownian rate of evolution; α = strength of attraction towards θ; θ = optimal trait value; θ_D, θ_H, θ_S = optimal trait value for Dry, Humid or Seasonal species; OUM = OU model with multiple optima from `OUwie`; OU1 = OU model with single optimum from `OUwie`. The lowest AIC model for each trait is marked in **bold**.

3.4 Phylogenetic regressions

The phylogenetic PCA that was performed to reduce dimensionality in the 19 bioclimatic variables resulted in three retained climate PCs, which were added to the data set. The loadings of these PCs are given in Table 3.5; a biplot of the first two PCs is displayed in Figure 3.6. All three retained PCs jointly explained 79% of the variance in bioclimatic variables; PC1 and PC2 explained 35% and 31% of the variance, respectively. The strongest loadings for PC1 were all highly positive: MTDRYQ (0.97), MINTCM (0.96), MTCOLDQ (0.95), and AMTEMP (0.93) (Abbreviations are explained in Table 2.2). PC1 therefore represents a general axis of temperature, with strongly positive values representing high temperatures. PC2 displayed the strongest loadings negatively for APREC (-0.91), PDRYQ (-0.86), PDRYM (-0.85), and PWARMQ (-0.78). This axis thus generally represents an axis of precipitation (or rather, drought), with high positive values indicating less rainfall. For PC3, the strongest loadings were TEMPSEAS (+0.74), ISOTHER (-0.71), and TEMPAR (+0.52). We interpret this PC as an axis of temperature seasonality, with strong positive scores indicating strong seasonal temperature fluctuations, and strong negative scores representing a constant temperature year-round.

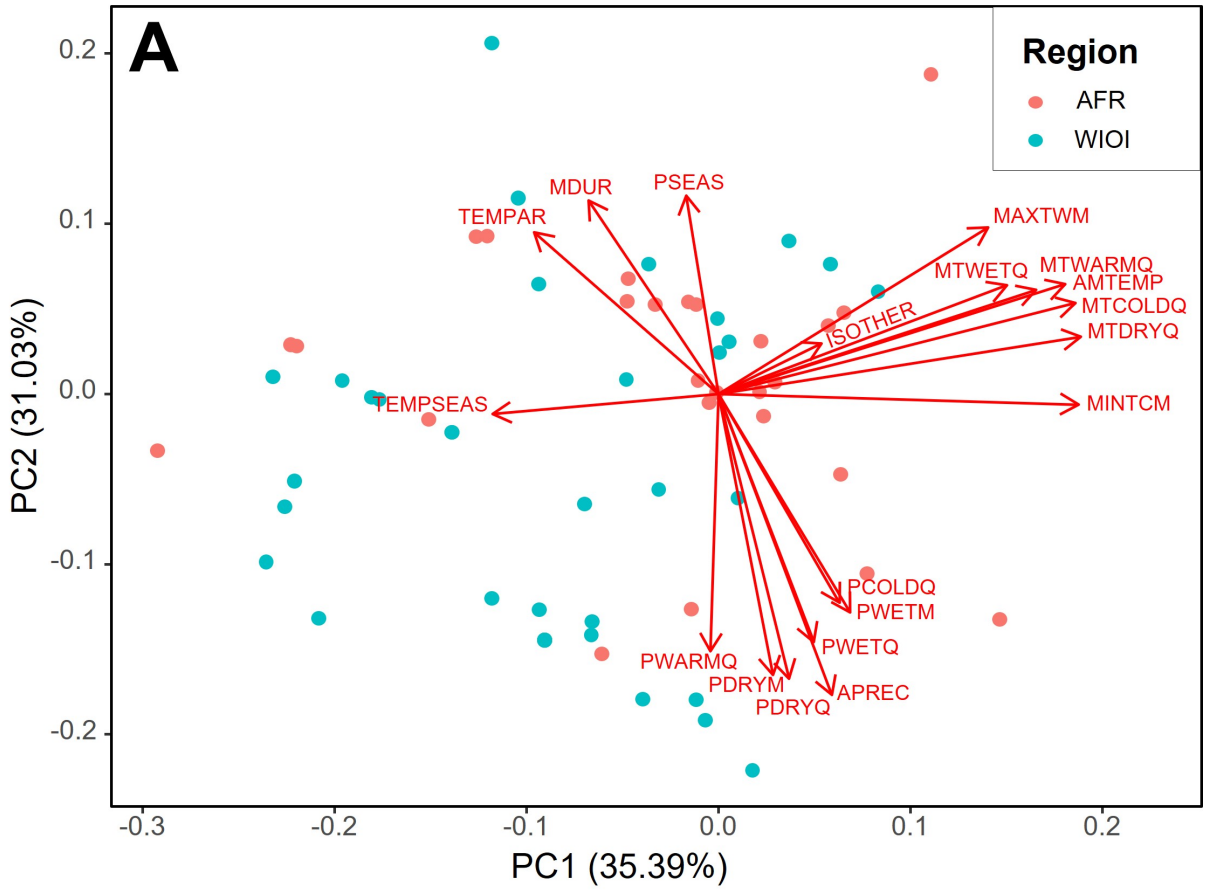
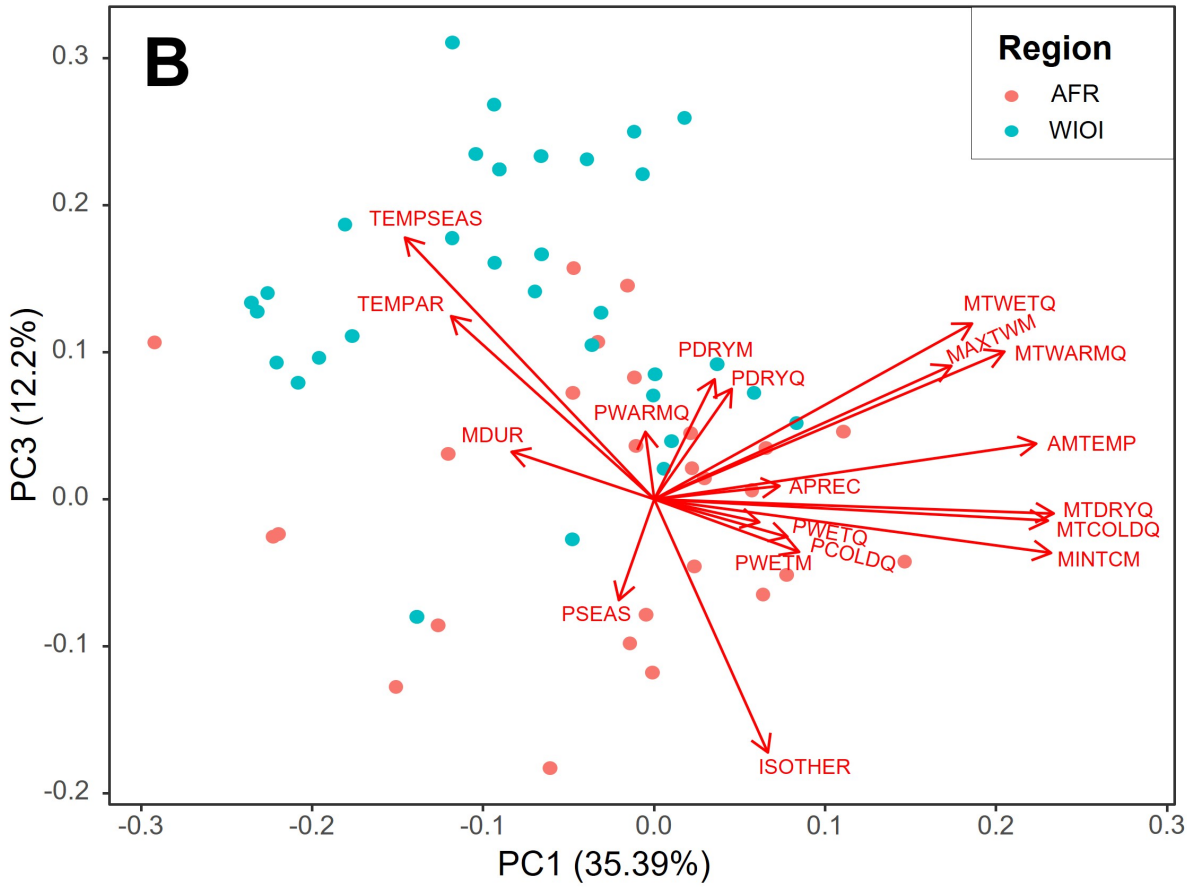


Figure 3.6: Biplots showing the retained PCs of the bioclimatic variable phylogenetic PCA. **A**: PC1 versus PC2; **B**: PC1 versus PC3. Arrows indicate loadings; points indicate species. Species are colored based on occurrence in continental Africa (AFR) or the West Indian Ocean Islands (WIOI). (Continued on next page)



(Continued) Biplots showing the retained PCs of the bioclimatic variable phylogenetic PCA. **A**: PC1 versus PC2; **B**: PC1 versus PC3. Arrows indicate loadings; points indicate species. Species are colored based on occurrence in continental Africa (AFR) or the West Indian Ocean Islands (WIOI).

For all traits, the multiple pGLS regressions revealed significant correlations with other variables in the data set (Tables 3.6-3.11). Note that non-significant predictors were sometimes retained in the models due to the fact that their removal increased the model AIC (*i.e.*, worsened the relative model fit). Also note that different traits are expressed in different units. Regression coefficients were not standardized, and therefore represent the change in the dependent variable for a change of 1 in the unit of the independent variable. The model was not able to estimate a lower bound for the 95% Confidence Interval (CI) of λ in any of the fitted regressions. Since the ML estimation of λ in the models is bound at zero, this likely reflects a negative lower bound estimate of the CI (which therefore includes zero). Unlike the univariate estimates for λ (Table 3.2), five of the six pGLS regressions obtained ML estimates of $\lambda = 0$, and thus did not account for phylogenetic signal. Only the model for leaf area took phylogenetic signal into account: the ML estimate for λ was 0.464 (95% CI: (NA, 0.906)).

Table 3.5: Loadings, (cumulative) variance explained, and eigenvalues for the three PC axes retained from the phylogenetic PCA of the 19 bioclimatic variables.

	PC1	**PC2**	**PC3**
AMTEMP	0.93	0.33	0.16
MDUR	-0.35	0.58	0.13
ISOTHER	0.28	0.15	-0.71
TEMPSEAS	-0.60	-0.06	0.74
MAXTWM	0.72	0.50	0.38
MINTCM	0.96	-0.03	-0.15
TEMPAR	-0.49	0.49	0.52
MTWETQ	0.77	0.33	0.50
MTDRYQ	0.97	0.17	-0.04
MTWARMQ	0.85	0.31	0.42
MTCOLDQ	0.95	0.27	-0.06
APREC	0.30	-0.91	0.04
PWETM	0.35	-0.66	-0.15
PDRYM	0.15	-0.85	0.34
PSEAS	-0.09	0.60	-0.28
PWETQ	0.26	-0.75	-0.06
PDRYQ	0.19	-0.86	0.31
PWARMQ	-0.02	-0.78	0.19
PCOLDQ	0.32	-0.63	-0.11
Variance explained	0.35	0.31	0.12
Cumulative variance	0.35	0.66	0.79
Eigenvalue	6.72	5.89	2.32

Definitions for the abbreviated bioclimatic variables are given in Table 2.2.

Stomatal length ($P < 0.001$) and leaf area ($P = 0.015$) were found to be significant negative and positive predictors of stomatal density, respectively. A negative effect of SLA on stomatal density was marginally significant, and climate or other stomatal dimensions had no significant effect (Table 3.6). Species with shorter stomata or larger leaves thus tended to have more stomata per unit of leaf area.

The effect of stomatal density on leaf area was significantly positive ($P = 0.038$). Latitude also had a significantly positive effect on leaf area ($P = 0.035$), whereas climate PC2 was a significant negative predictor ($P = 0.002$). The effects of pore width ($P = 0.076$) and climate PC3 ($P = 0.067$) were positive, but only marginally significant (Table 3.7). Leaves in the *Coffea* genus were thus generally larger at higher latitudes, and in climates with more precipitation. Larger leaves also have more stomata per mm^2. Larger leaves might also tend to have wider pores, and leaves may be larger in climates with more seasonal temperature variability.

The regression with SLA as the dependent variable resulted in two significant negative predictors: leaf area ($P = 0.046$) and longitude ($P = 0.024$) (Table 3.8). The positive effects of altitude ($P = 0.072$) and climate PC1 ($P = 0.088$) approached significance. Species sampled further east therefore tended to have slightly lower SLA than more western species. *Coffea* species with larger leaves also generally had lower SLA values. There may also be a trend for species in warmer climates to have a higher SLA.

Stomatal length was significantly positively predicted by stomatal width ($P < 0.001$) and pore width ($P = 0.006$). The negative effect of stomatal density was also highly significant ($P < 0.001$) (Table 3.9). Species with wider stomata and stomatal pores thus also tended to have longer stomata, and high stomatal densities are associated with lower stomatal length.

Unlike stomatal length, stomatal width was not significantly predicted by pore width. However, stomatal length ($P < 0.001$) and leaf area ($P = 0.043$) were both significant positive predictors of stomatal width (Table 3.10). Longer stomata thus tended to be wider, and species with larger leaves also tended to have wider stomata.

Variation in pore width was significantly explained by only a single independent variable: stomatal length ($P < 0.001$), which had a positive effect on pore width (Table 3.11). Species with longer stomata thus also displayed wider pore openings.

Table 3.6: Multiple pGLS regression taking stomatal density as the dependent variable, and all other considered variables as independent variables.

Stomatal density	**Coefficient**	**SE**	***t* value**	***P***	
(**Intercept**)	571.31	72.49	7.88	<0.001	***
log(Leaf area)	20.26	8.00	2.53	0.015	*
SLA	-3.01	1.65	-1.82	0.074	.
Stomatal length	-12.13	2.34	-5.19	<0.001	***
$\lambda = 0$ (NA, 0.784)	$R^2_{adj} = 0.38$			AIC = 599.47	

For λ, the values between brackets indicate the 95% confidence interval. R^2_{adj} = Adjusted R^2. Significance: . , $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 3.7: Multiple pGLS regression taking log(Leaf area) as the dependent variable, and all other considered variables as independent variables.

log(Leaf area)	**Coefficient**	**SE**	***t* value**	***P***	
(**Intercept**)	-7.96	0.74	-10.81	<0.001	***
Stomatal density	3.42E-3	1.61E-3	2.13	0.038	*
Pore width	0.31	0.17	1.81	0.076	.
Latitude	4.80E-2	2.21E-2	2.17	0.035	*
Climate PC2	-0.19	5.89E-2	-3.29	0.002	**
Climate PC3	0.22	0.12	1.87	0.067	.
$\lambda = 0.464$ (NA, 0.906)	$R^2_{adj} = 0.29$			AIC = 133.77	

For λ, the values between brackets indicate the 95% confidence interval. R^2_{adj} = Adjusted R^2. Significance: . , $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 3.8: Multiple pGLS regression taking SLA as the dependent variable, and all other considered variables as independent variables.

SLA	**Coefficient**	**SE**	***t* value**	***P***	
(Intercept)	4.60	6.92	0.67	0.509	
log(Leaf area)	-1.44	0.70	-2.05	0.046	*
Stomatal length	-0.15	0.19	-0.79	0.432	
Longitude	-0.11	0.05	-2.34	0.024	*
Altitude	1.07E-2	5.84E-3	1.84	0.072	.
Climate PC1	1.71	0.98	1.74	0.088	.
Climate PC2	-0.49	0.35	-1.39	0.170	
Climate PC3	1.47	1.07	1.37	0.177	
$\lambda = 0$ (NA, 0.635)	$R^2_{adj} = 0.18$			AIC = 318.55	

For λ, the values between brackets indicate the 95% confidence interval. R^2_{adj} = Adjusted R^2. Significance: . , $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 3.9: Multiple pGLS regression taking stomatal length as the dependent variable, and all other considered variables as independent variables.

Stomatal length	**Coefficient**	**SE**	***t* value**	***P***	
(**Intercept**)	7.41	1.98	3.73	<0.001	***
Stomatal density	-1.26E-2	3.23E-3	-3.89	<0.001	***
Stomatal width	0.85	0.11	7.78	<0.001	***
Pore width	1.13	0.39	2.89	0.006	**
Longitude	-1.85E-2	1.30E-2	-1.43	0.160	
$\lambda = 0$ (NA, 0.380)	$R^2_{adj} = 0.78$			AIC = 206.83	

For λ, the values between brackets indicate the 95% confidence interval. R^2_{adj} = Adjusted R^2. Significance: . , $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 3.10: Multiple pGLS regression taking stomatal width as the dependent variable, and all other considered variables as independent variables.

Stomatal width	**Coefficient**	**SE**	***t* value**	***P***	
(**Intercept**)	5.10	1.69	3.01	0.004	**
log(Leaf area)	0.39	0.19	2.08	0.043	*
Stomatal length	0.58	5.37E-2	10.71	<0.001	***
Climate PC3	0.21	0.13	1.67	0.100	
$\lambda = 0$ (NA, 0.169)	$R^2_{adj} = 0.69$			AIC = 185.53	

For λ, the values between brackets indicate the 95% confidence interval. R^2_{adj} = Adjusted R^2. Significance: . , $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 3.11: Multiple pGLS regression taking pore width as the dependent variable, and all other considered variables as independent variables.

Pore width	**Coefficient**	**SE**	***t* value**	***P***	
(**Intercept**)	1.57	0.65	2.40	0.020	*
log(Leaf area)	0.11	7.25E-2	1.49	0.141	
Stomatal length	0.11	2.08E-2	5.15	<0.001	***
$\lambda = 0$ (NA, 0.172)	$R^2_{adj} = 0.34$			AIC = 81.43	

For λ, the values between brackets indicate the 95% confidence interval. R^2_{adj} = Adjusted R^2. Significance: . , $P < 0.10$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Chapter 4

Discussion

A better comprehension of the variation in functional leaf traits within *Coffea*, as well as disentangling how these traits relate to each other and to climate, is essential for understanding the adaptive value of these traits throughout the genus. Furthermore, investigating how these traits may have evolved over time can yield useful information for future conservation and crop improvement strategies. The goal of this study was to assess the trait variation present between and among *Coffea* species, identify correlations with other traits or climate variables, and gain insight into the evolutionary processes that may have shaped the present trait diversity in the *Coffea* genus. By employing Phylogenetic Comparative Methods and models of continuous trait evolution, we investigated leaf trait variation and evolution that had not been previously examined at the genus level. We detected a number of associations between traits, as well as some trait-climate correlations. Our analyses also provide useful insight into the drivers of functional leaf trait evolution in *Coffea*.

4.1 Leaf trait variation and integration

The analyses performed in this study contribute to the mapping of the trait variation present in the *Coffea* genus. It is clear from the data that there is considerable variation in the measured trait values, both within and between species. Though the majority of the variance is explained by between-species differences, some species show considerable intraspecific variation in certain traits. The variation detected in this study likely still greatly underestimates the trait variation present in wild populations, because our samples were limited to herbarium specimens. However, the observation that between-species differences contribute more to the variance than intraspecific differences remains valid, and should be applicable to natural situations as well. This pattern indicates that species in the *Coffea* genus have diverged significantly in their morphological leaf traits over evolutionary time.

The first two PC axes from the leaf trait phylogenetic PCA show that certain leaf traits are clearly integrated with each other across the studied *Coffea* species, and have evolved in a modular fashion. The first axis, with strong loadings for stomatal dimensions and stomatal density, explained almost twice the amount of variance as PC2. The observation that stomatal dimensions have opposite loadings to stomatal density along PC1 indicates that species with larger stomata tend to have lower stomatal densities throughout the genus. This is confirmed

by the results of our pGLS regression analyses, where stomatal dimensions generally correlated positively with each other, but negatively with stomatal density. This conclusion is in agreement with our expectations, as well as with the findings of Brodribb et al. (2013) in the Proteaceae family. They concluded that guard cell size correlated positively with other plant cell sizes, but negatively with stomatal density and leaf vein density (Brodribb et al., 2013). Indeed, the trade-off between stomatal size and stomatal density has been observed various other times in literature (*e.g.*, Drake et al. (2013); Hetherington and Woodward (2003); Franks and Beerling (2009)).

Stomatal size and density

The mechanisms of the trade-off between stomatal size and stomatal density can tell us more about the implications of these observations. Smaller stomata have been proposed to respond more rapidly to environmental conditions (Hetherington and Woodward, 2003; Drake et al., 2013), though Bertolino et al. (2019) claim that this is unlikely to affect water loss during longer periods of drought. The combination of small stomata with high stomatal densities is associated with higher stomatal conductance, which regulates both CO_2 uptake and water loss through transpiration (Franks and Beerling, 2009). At low CO_2 concentrations, high densities of small stomata are required to extract the largest amount of CO_2 from the air and avoid CO_2 starvation. However, stomatal conductance has been found to be negatively correlated with WUE across species: high gas exchange inevitably leads to more water loss (Drake et al., 2013). This would imply that low densities of large stomata should lead to improved WUE. Nonetheless, other research, has shown positive correlations between stomatal density and WUE, because photosynthetic capacity increases more than water loss with increasing stomatal density (Xu and Zhou, 2008). Growth under water deficit, where WUE is critical, has indeed been found to decrease stomatal size and increase stomatal density (Xu and Zhou, 2008; Doheny-Adams et al., 2012). Results across species and environments thus remain conflicting, and these relationships should be assessed independently for a given taxon (Bertolino et al., 2019).

In the case of *Coffea*, our trait PCA shows that continental African species tend to have lower densities of larger stomata than WIOI species. This apparent trait divergence between the two regions likely represents two different sides of the functional trade-off described above. Based on our hypotheses, we would expect this trait divergence to be a result of differing climatic regimes in continental Africa and the WIOI islands. At a global level, increasing levels of habitat aridity can be expected to associate with smaller leaves and lower stomatal conductance (*i.e.*, lower densities of larger stomata) (Farquhar et al., 2002; Franks and Beerling, 2009; Thuiller et al., 2004). These conditions would selectively favor plants with higher WUE, thus having larger stomata and lower stomatal densities according to our hypothesis. However, our analyses show no obvious distinction in climate PC1 or PC2 between mainland and island species, despite the fact that mainland species were sampled across a broader geographical range. Only climate PC3 seems to show a divergence between the two regions, with island species experiencing greater temperature fluctuations throughout the year. In a drought adaptation scenario, where fewer, larger stomata are an adaptation to increased habitat aridity, we would expect mainland species to show higher climate PC2 values than WIOI species. However, no such pattern is apparent in our data. Moreover, the pGLS regressions revealed no correlations between stomatal size or density and climate. It is possible that this trait

divergence between the two regions is simply the result of geographic isolation and genetic drift, or that these traits were selected for in response to other environmental variables not considered here, such as soil nutrient content or biotic interactions.

Leaf area

Although leaf area is known to vary broadly within climates or latitude ranges, climate is generally a strong determinant of leaf size across species (Wright et al., 2017). Leaf size has a direct impact on functional processes in the leaf, such as water balance or photosynthesis (Givnish, 1987; Blonder et al., 2012). Contrasting with our results for stomatal size and density, the finding that climate PC2 (our axis of habitat aridity) was a significant negative predictor of leaf area aligned with our expectations. We hypothesized that more arid areas would select for smaller leaf sizes to limit water loss via transpiration (Thuiller et al., 2004; Poole and Miller, 1981; Wright et al., 2017). This correlation reflects a functional trade-off between drought tolerance and productivity, given that smaller leaves provide less surface area to intercept light for photosynthesis (Thuiller et al., 2004).

In addition to habitat drought, leaf area displayed a significant positive correlation with latitude. Since our samples were collected roughly between 25°S and 10°N, it is reasonable to state that species closer to the equator tended to have larger leaves than species further south. On the African continent, more humid tropical rainforests occupy most of the equatorial region, with deciduous woodlands and savannah biomes towards the south and the east (White, 1983). This result is therefore in agreement with our expectations: tropical rainforest species tend to have larger leaves than species from the more southern woodlands or savannahs. This is consistent with the negative correlation between climate PC2 and leaf area, and with the global trend shown by Wright et al. (2017), who found that leaf size is largest at the equator across species worldwide. Furthermore, the pGLS regression also suggests a possible role for leaf area in adapting to seasonal temperature variations, showing a marginally significant correlation between leaf area and climate PC3.

Despite substantial previous research, it is still not entirely clear which climate characteristic leaf size responds to the most: temperature or precipitation (Mitchell et al., 2018; Thuiller et al., 2004; Moles et al., 2014; Poole and Miller, 1981). In a global study across diverse vegetation types and climates that included over 25 thousand species, Moles et al. (2014) determined that mean annual temperature was more strongly correlated with plant traits than mean annual precipitation. The effect on leaf area, specifically, was also stronger for temperature than it was for precipitation. However, studies in specific clades have found contrasting results. For example, Thuiller et al. (2004) examined plant trait distributions across climate gradients in the genus *Leucadendron*, a prominent taxon in the Cape Floristic region. They found that aridity, not temperature, significantly influenced leaf area. Poole and Miller (1981) also found leaf area to increase with precipitation in the chaparral of southern California. Mitchell et al. (2018), on the other hand, found leaf area to correlate positively with temperature, and not with precipitation. It is obvious that temperature and precipitation are not independent of each other, and that, for example, hot and dry environments will exert different pressures on plants than hot and humid climates (Wright et al., 2017). Furthermore, it is likely that despite global patterns, different climatic aspects will have different impacts across the plant kingdom (Mitchell et al., 2018). These relationships should therefore be evaluated separately in different families or genera. We detected no significant association between leaf

size and temperature, whereas the precipitation PC had a very significant effect. There was, however, a marginally significant positive relationship between seasonal temperature variability and leaf area. This indicates that leaf size responds more to precipitation than to temperature in the *Coffea* genus, but seasonal temperature fluctuations may also play a role in determining leaf size. According to Wright et al. (2017), temperature and precipitation interact with each other in their effect on leaf size. We observed no such effect in the *Coffea* species studied here, since our climate PC was not included in the best-fitting model.

Surprisingly, leaf area was also positively correlated with stomatal density. If a high density of small stomata minimizes water loss, we would not expect this positive correlation. Consequently, species with larger leaves have a much greater total amount of stomata per leaf. In combination with the marginally significant positive correlation between leaf area and pore width, this points to a much higher stomatal conductance in larger leaves across the *Coffea* genus. Species enduring less drought stress therefore have larger leaves, and accordingly also higher stomatal densities and possibly larger stomata, allowing them to maximize their carbon assimilation when water is not limiting. Apparently, the observed negative correlation between stomatal density and stomatal size is at least partially dependent on leaf area. Our observations therefore suggest that high densities of large stomata provide the highest gas exchange capacity, and that this trait combination is thus adaptive in humid environments when water is not limiting (and vice-versa for dry environments). Due to physical restrictions, however, leaf size needs to increase to allow an increase in both stomatal density and stomatal size.

The relationship between stomatal traits and climate may thus depend on other factors, such as leaf size or other unmeasured traits or environmental variables. Although we detected no direct effect of aridity on stomatal size or density, it is possible that changes in leaf area in response to dryness could compensate to a certain extent for this lack of effect. Theoretical work by Farquhar et al. (2002) has suggested that the magnitude of change in stomatal conductance in response to water availability is diminished by changes in leaf area. It is therefore possible that evolutionary change in stomatal characteristics is simply overshadowed by changes in leaf area, or that selection on leaf area is more efficient than selection on stomatal traits. The positive correlation between stomatal density and leaf area, but not between stomatal density and climate, supports this interpretation. Therefore, we propose that the selection pressure of drought stress acts more on leaf area than it does on stomatal traits in the *Coffea* genus, and that changes in leaf area in response to drought stress compensate for a relative lack of change in stomatal conductance (*i.e.*, stomatal density and stomatal size).

SLA

One of the fundamental traits integrated in the Leaf Economics Spectrum (LES) is LMA, the inverse of SLA (Wright et al., 2004). The LES can therefore serve as a framework to help interpret correlations observed in our trait data. According to the LES, low LMA (*i.e.*, high SLA) generally implies a high photosynthetic capacity, high respiration rate, low leaf lifespan, and high leaf nutrient concentrations, *i.e.*, a generally fast-paced ecological strategy (Wright et al., 2004; Reich, 2014). Species with these traits have the potential to get very fast returns on their investments in leaf biomass, whereas the opposite is true for species at the other end of the LES (Wright et al., 2004).

In our data, we observed that SLA was significantly negatively correlated with longitude

across the full range of *Coffea* sampling locations. Species occurring at more eastern longitudes (thus generally WIOI species) tend to have lower SLA values than western (continental African) species. Moreover, the trait phylogenetic PCA shows some distinction between continental African and WIOI species along the loading vector for SLA, with mainland species having higher SLA values. Whether this relationship between longitude and SLA holds within each region, however, was not tested explicitly. The combination of these observations implies that mainland species exhibit a slightly "faster" ecological strategy than island species, *i.e.*, a resource-acquisitive strategy with low investment in leaf dry mass and potentially shorter leaf lifespans (Wright et al., 2004; Read et al., 2014). The marginally significant positive correlation between climate PC1 and SLA may suggest that this faster strategy is due to higher temperatures on the African continent, though no clear climatic divergence between both regions was obvious from the PCA.

In theory, any given species adopting a fast or slow strategy should do so consistently across all traits (Reich, 2014). We would therefore expect species with "fast" traits, such as high SLA, to also exhibit uniformly fast strategies in their stomatal traits. It could be argued that having many, small stomata represents a fast ecological strategy, because of the higher stomatal conductance these traits provide (Franks and Beerling, 2009; Drake et al., 2013). Few, large stomata, on the other hand, would then be indicative of a slower strategy, where CO_2 uptake is reduced (Drake et al., 2013). The observation that mainland species have lower densities of larger stomata than WIOI species therefore suggests a "slower" ecological strategy on the continent, which contrasts with the hypothesis of Reich (2014), that traits should be uniformly fast or slow for the whole plant in any given taxon. However, as shown above, it is still unclear to what extent the trade-off between stomatal size and density represents contrasting adaptive strategies in *Coffea*.

Further, one might expect SLA to be inversely correlated to climate PC2 as a response to drought stress. Previous research has shown negative correlations between SLA and WUE, for example in peanuts (Craufurd et al., 1999) or in grass species (Xu and Zhou, 2008). Also, according to Mitchell et al. (2018), less rainfall should be associated with traits that reflect a "slower" life-history strategy. With less precipitation, plants are expected to invest more in their tissues, leading, for example, to lower SLA. We did not observe this correlation in our data: PC2 was included in the best-fitting model for SLA, but the effect was non-significant. However, the significant positive correlation between SLA and leaf area (which is negatively correlated with climate PC2) does align indirectly with this expectation. In addition to the negative correlation between SLA and WUE, Xu and Zhou (2008) also experimentally detected a negative correlation between SLA and stomatal density in the perennial grass species *Leymus chinensis*, sampled from the Mongolian steppe. We also detected a negative effect of SLA on stomatal density across the *Coffea* genus, but the effect was only marginally significant.

Though SLA and leaf area may appear to be similar metrics, the data shows that they are slightly negatively correlated. Larger leaves thus have a tendency to be more dense in the *Coffea* genus, although the correlation was only slightly significant. The relationship between leaf area and SLA has been found to vary depending on the scale at which it is assessed (Ackerly et al., 2002). Moreover, WIOI species on average have lower SLA (judging by the the trait PCA and the negative correlation between longitude and SLA), but do not show markedly higher values for climate PC2 (*i.e.*, less rainfall). Therefore, it is clear that SLA does not exhibit the same correlation patterns as leaf area. In a study on 22 chaparral shrub

species, leaf size was highly positively correlated with SLA at the community level. However, this relationship did not hold across species, where the traits became fully uncoupled. On the other hand, Onstein et al. (2016) did detect a strong positive correlation between leaf area and SLA across the Proteaceae family. Our results reinforce the conclusion of Ackerly et al. (2002) that these traits influence different aspects of plant performance across environmental gradients, and can therefore vary relatively independently from each other (Ackerly et al., 2002).

Other patterns

Aside from direct trait-trait or trait-climate correlations, our analyses show a number of other noteworthy patterns in the data. Interestingly, no significant effects of altitude on any of the measured traits were detected, despite the wide range of altitudes across sampling sites in the data set (roughly between 0 and 1500m; see Supplementary material S2). Many environmental factors are known to covary with altitude: higher altitudes are generally associated with lower temperatures due to adiabatic cooling (Mitchell et al., 2018), and resource availability is often lower at higher elevations (Read et al., 2014; Körner, 1989). Following the reasoning of Read et al. (2014) and Körner (1989), we would expect plants at higher elevations to exhibit more stress tolerance and higher tissue investment, *i.e.*, a "slower" ecological strategy with low SLA.

Notwithstanding the disparity with our initial expectations, this lack of altitudinal effects on trait values is consistent with our other results. Since we detected no significant effects of temperature, it is reasonable to expect that altitude would not significantly affect the tested traits either. However, altitude did show a marginally significant positive correlation with SLA, possibly suggesting lower leaf investment and a faster ecological strategy at higher altitudes. This result is opposite to our hypothesis, and to the findings of Read et al. (2014). This may call for further research into the patterns of functional leaf traits across elevation gradients in *Coffea*.

Overall, climate PCs were only included in the best-fitting pGLS model for three out of the six studied leaf traits: leaf area, SLA, and stomatal width. Moreover, only one significant correlation between climate and leaf traits was detected (the effect of climate PC2 on leaf area), although some other effects approached significance. These findings support our initial hypothesis that correlations between traits and climate variables would be relatively weak, as informed by previous research (Moles et al., 2005; Vandelook et al., 2012; Wright et al., 2004). It is known that other factors, such as light and nutrient availability, also influence trait variability in *Coffea* (Buchanan et al., 2019). However, these factors each explained relatively small amounts of variation in the data. A multitude of factors most likely contributes in small part to leaf trait variation, including but not limited to climate factors (*e.g.*, Wright et al. (2017)), irradiance (*e.g.*, Farquhar et al. (2002); Drake et al. (2013)), soil properties (*e.g.*, Buchanan et al. (2019)) or biotic interactions (*e.g.*, Chapin et al. (1997); Givnish (1984)).

4.2 Trait evolutionary history

To investigate and contextualize the evolutionary history of *Coffea* leaf traits, we estimated univariate and multivariate phylogenetic signal, and fitted evolutionary models to the trait data.

The pGLS regressions, which accounted for the effects of other traits and climate in the estimation of λ, did not detect significant phylogenetic signal in any of the studied leaf traits. This lack of signal was not entirely unexpected, considering the fact that OU models were the best fit to the data for all traits. An intrinsic property of an OU process is that evolutionary history becomes gradually less important over time, reducing phylogenetic signal (Felsenstein, 1988; Blomberg et al., 2003). In an OU model of evolution, a continuous trait value will fluctuate around a central optimum, with recent stochasticity causing most of the trait variation. The attraction to the optimal trait value thus progressively erases the influence of older character states (Felsenstein, 1988). The absence of phylogenetic signal is therefore a logical result if traits have evolved according to an OU process. Also, leaf traits are typically more susceptible to environmental fluctuations and can thus be expected to adapt rapidly to new environmental conditions, whereas seed and floral traits tend to be more evolutionarily conserved (Ackerly, 2009; Vandelook et al., 2018). However, this lack of signal can at least partially also be attributed to the small number of species in our phylogeny and to measurement error, both of which are known to obscure phylogenetic signal (Blomberg et al., 2003). The fact that the pGLS models were unable to estimate lower bounds for λ might also be partially attributed to the low sample size used. Likelihood profiles displayed relatively flat plateaus for all traits, making it difficult for the model to obtain an ML estimate (see Figure C.1 in Appendix C). These profiles also show that the ML estimate for leaf area ($\lambda = 0.464$) is likely not a meaningful value, since other values between 0 and 0.9 have nearly equal likelihoods.

The phylomorphospace plots of the trait PCA show that more basal species, such as the included outgroup species, the XC clade, and *C. charrieriana*, tend to have large stomata, along with low stomatal densities. This suggests that small stomata and high stomatal density are derived characteristics in the *Coffea* genus. The univariate phylogenetic signal estimates provide some additional support for this interpretation. Indeed, the λ estimates show significant phylogenetic signal in stomatal length and width across the full genus and its outgroups, although signal in stomatal width became non-significant when outgroups were removed from the data. The absence of signal in stomatal density, however, provides no further evidence to support the conclusion that low densities of large stomata is a basal trait combination. Although lower stomatal densities do appear to occur mainly in the basal branches of the phylogeny, there was no significant phylogenetic signal in the full tree. Within the WIOI subclade, the significant signal in stomatal density actually shows the opposite pattern, with the more basal branches of the clade having higher stomatal densities than the more recently derived taxa.

The most basal species of the *Coffea* genus (excluding outgroups) also appear to have low values for trait PC2 based on the phylomorphospace plots, *i.e.*, have high SLA values and lower leaf area and stomatal density. Again, this may indicate that high SLA is an ancestral characteristic in the genus, and that more derived species evolved towards lower values of SLA. This is corroborated by the presence of univariate phylogenetic signal in SLA. More basal species tend to have higher SLA values, whereas the most recently derived species

exhibit low SLA. Regarding leaf area and stomatal density, we cannot confirm this evolutionary trajectory because we detected no phylogenetic signal in these traits. As a whole, however, this exploration of the data suggests that *Coffea* species evolved and diversified on average towards higher leaf investment, small stomata and higher stomatal densities.

Interestingly, our data shows that two closely related species (*C. mauritiana* and *C. macrocarpa*) are very far apart in trait space, and thus have remarkably different leaf traits. Together, these species make up the most basal branch of the WIOI subclade and both inhabit humid environments. Despite their close phylogenetic relatedness, these species occur on different islands in the Indian Ocean: *C. macrocarpa* is endemic to Mauritius (Nowak et al., 2014), whereas our *C. mauritiana* accession was sampled on the neighboring island of Réunion. This strong divergence suggests that the strong isolation and novel niche availability following dispersal led to rapid trait evolution and divergence between both species. *C. mauritiana* has exceptionally small stomata, whereas *C. macrocarpa* has a very low average SLA.

In all cases, the OU model outperformed both the BM and EB models of trait evolution. In the two cases where the fit of the OU model was only marginally better than the other models, the EB model always had the worst fit (*i.e.*, stomatal length and stomatal width in the AFR subclade). We can therefore reasonably exclude an Early Burst pattern of evolution in the *Coffea* genus. Previous research has pointed towards adaptive radiation in the genus based on plastid DNA sequence data (Anthony et al., 2010). Our modelling approach did not provide any support to their findings, since the EB model, simulating adaptive radiation, was rejected for all tested clades. However, as progressively smaller clades are considered, sample size becomes smaller and modelling approaches can become biased (Cooper et al., 2016). We can therefore not conclusively exclude the possibility that trait radiation may be adaptive in the *Coffea* genus or any of its subclades.

The OU models obtained estimates for α that were notably high, and $t_{1/2}$ was therefore short relative to the considered phylogenies. Low half-life implies rapid trait change towards the evolutionary optimum for all considered traits. The effect of phylogenetic relatedness on trait similarity is therefore limited to species that are very closely related (Pan et al., 2014). Across the tested clades, stomatal density and pore width consistently exhibited the fastest trait change towards the optimum, with a half-life of less than 1% in all clades except the WIOI subclade. Stomatal length always displayed the slowest trait change in each clade, approaching a half-life of 30% in the AFR subclade. The influence of ancestral trait values is thus the greatest for stomatal length (Pan et al., 2014; Revell et al., 2008). The other three traits exhibited intermediate half-lives and α values.

Our main conclusion from the model comparisons is that the diversification of the six measured leaf traits was driven by more than purely genetic drift. The fact that α is consistently greater than zero, and that λ is consistently smaller than 1, implies that the evolution of these traits has not been a pure drift process (Vandelook et al., 2012). Our models suggest a substantial role for phylogenetic niche conservatism in shaping the existing variation in the *Coffea* genus for the traits considered here. The range of variation in trait values is therefore bounded to a certain range. However, this does not directly imply the presence of an adaptive process or stabilizing selection, only that there is a degree of evolutionary stasis in these traits as compared to what one might expect under a BM (pure drift) model of evolution. Other possible causes of phylogenetic niche conservatism are gene flow or genetic constraints due to pleiotropy, both preventing adaptation to new environments, or simply

insufficient standing genetic variation for adaptation (Losos, 2008; Wiens and Graham, 2005; Mitchell et al., 2018). The high α values, alongside the strong trait divergence between *C. macrocarpa* and *C. mauritiana*, indicates that leaf traits are capable of adapting rapidly to novel environments. We therefore consider it unlikely that pleiotropy or lack of standing variation play a large role in determining the distribution of leaf traits across the *Coffea* genus. Similarly, we assume that effects of gene flow are negligible between different species. The most plausible interpretation for the patterns observed here is that continuous stabilizing selection leads to ecological similarity among closely related species.

Optimal trait values for stomatal density differed substantially between clades (161.22 stomata per mm^2 in the AFR subclade versus 186.79 stomata per mm^2 in the WIOI subclade), though the significance of these differences was not tested. All other measured traits seemed to have relatively consistent θ values across clades. This indicates that different selection pressures may be at play in the different branches of the phylogeny, leading to differences in evolutionary optima for stomatal density between the clades. This should be further investigated by fitting an OUM model with different optima for each clade, and comparing the fit of this model to other models such as the ones fitted here.

An interesting observation is the fact that the OUM models were a better fit than the OU1 models for leaf area and stomatal width. This signals that these traits have evolved towards different optima in different levels of habitat dryness, and provides more support to the interpretation that these traits may indeed be adaptive in nature. In combination with the α values obtained for these traits, this result supports the hypothesis that these traits are shaped and maintained by stabilizing selection. The short $t_{1/2}$ also indicates that evolution toward these climate-specific optima is rapid (Vandelook et al., 2012). For leaf area, the θ values align with what one might expect based on previously observed trait-climate relationships (Poole and Miller, 1981; Thuiller et al., 2004): leaf size in species in humid environments evolves around a higher optimum than species from seasonal or dry environments. The differential optima for stomatal width are less intuitive to explain: seasonal environments exhibit the smallest optimal stomatal width, whereas dry and humid environments differ very little in their optima. As is widely known, stomatal width and pore width are dependent on the current state of stomatal aperture. However, all our stomatal measurements were performed on herbarium specimens. We assumed these specimens to be equally dry, thus with all stomata in a similar state of aperture. Nonetheless, this dependence could potentially obscure patterns in the trait data. Also, the outperformance of the OUM model compared to the OU1 model for stomatal width was only marginal (ΔAIC $= 0.58$). We therefore advise that the model results for stomatal width be interpreted with caution.

Though we did not test heritability of these traits directly, the high values for α estimated in the OU models suggest that these traits are strongly influenced by stabilizing selection. This strong pull towards the optimum implies a substantial role for genetic factors in maintaining the current trait diversity in the genus. Additionally, a 2021 study in *C. canephora* has indicated that phenotypic variation can be mainly attributed to genetic differences between phenotypes (Dubberstein et al., 2021). Their study looked at many of the same traits examined here, suggesting that sufficient heritability is present for these traits, and that artificial selection approaches should be viable. It is thus not unreasonable to assume that these traits would have a similar genetic basis in other *Coffea* species, such as the species investigated in this study.

4.3 Considerations for future research

While we are confident that our study presents some notable findings, we acknowledge some methodological issues in our work that may reduce confidence in the robustness of our results.

An overarching issue hindering the analyses performed in this study, is the relatively small number of species included. Though the *Coffea* genus includes 124 known species (Davis et al., 2011; Hamon et al., 2017), data for only 58 of these taxa was available from Meise Botanic Garden. According to Beaulieu et al. (2012), OU model parameters are generally overestimated with small data sets up to 128 species, which could contribute to our high estimates for α and σ^2. Cooper et al. (2016) also caution against fitting OU models to small phylogenetic trees, suggesting that trees with under 200 tips will often incorrectly favor the OU model over other models, such as BM or EB. Similarly, even small amounts of measurement error in the trait data have been shown to bias model selection in favor of OU models (Cooper et al., 2016). Though we account for standard error in the fitting of the evolutionary models, this led to extremely high AIC values in one case, most likely due to an overfitting problem. Other studies fitting similar models to evolutionary trees tend to use larger data sets than ours (*e.g.*, Onstein et al. (2016); Tonnabel et al. (2018); Turcotte et al. (2014); Vandelook et al. (2012)) or use Bayesian methods in an attempt to better predict parameters (*e.g.*, Vieu et al. (2021)). Using a Bayesian Markov Chain Monte Carlo (MCMC) approach has been suggested as a promising strategy for small phylogenies (Cooper et al., 2016), but these methods are much more computationally intensive and time-consuming than ML methods, and may be equally affected by parameter identifiability issues (Ho and Ané, 2014). Small sample sizes are also known to affect the uncertainty in Pagel's λ estimates, masking phylogenetic signal (Münkemüller et al., 2012; Blomberg et al., 2003). This can partially explain why our pGLS regressions were unable to provide reliable estimates for λ. Despite these possible biases, the difference in AIC values between the different models was generally large for most traits and clades, with OU models outperforming the other models by a sizable margin. We therefore remain confident that the results of the analyses performed here are meaningful. Nonetheless, we recommend that future research include data on a larger number of species in the genus, or that future studies should consider higher taxonomic levels. Whenever possible, we also propose the comparison of MCMC and ML methods for model comparison and parameter estimation, to determine which of the two is more appropriate.

In contrast to the evolutionary models, pGLS regressions are often conducted in literature with sample sizes that are similar to (or smaller than) ours (*e.g.*, McCormack et al. (2020); Corcobado et al. (2012); Mitchell et al. (2018)). Additionally, the Type I error rates of pGLS have been found to be appropriate for smaller phylogenetic trees than those used in this study (Adams and Collyer, 2018). Nonetheless, the trait correlations detected in our pGLS regressions should be interpreted with some caution. A number of unmeasured factors could potentially confound these correlations (*e.g.*, plant height, age, habitat shade) (Vandelook et al., 2018). This study focused specifically on leaf traits, and did not account for correlations with other traits at the whole-plant level. The pGLS regressions also assumed BM evolution, which we rejected in favor of OU models. Another possible confounding effect is that of measurement error in predictor variables, which was not incorporated into the pGLS models due to the substantial complexity of its implementation (Revell, 2012a).

Though our pGLS regressions detected only one significant trait-climate association (*i.e.*, between leaf area and precipitation), it is possible that the dimensionality reduction through PCA masked significant associations with individual bioclimatic variables. Put differently, our regressions might not detect a significant correlation of a trait with any individual bioclimatic variable because of confounding effects of other loadings in the PCs. However, it is not easily feasible to fit models with each individual bioclimatic variable as a predictor, due to the limited number of species in the genus. Though methods exist to deal with a large number of traits in a limited number of species (*e.g.*, Clavel et al. (2019)), these situations remain challenging. To gain additional information, however, future researchers may consider including interaction terms into the pGLS regressions. Our lowest AIC models often retained non-significant predictors, which could be indicative of interaction effects present in the independent variables.

When comparing the fit of multiple models, we chose to base our model selection on AIC values. However, multiple information criteria exist to inform model comparisons, such as the small-sample corrected Akaike Information Criterion (AICc) or the Bayesian Information Criterion (BIC). In short, AICc is equivalent to AIC but more accurate in cases where there ratio of sample size to number of parameters is low (Burnham and Anderson, 2002). In our data, we deemed the sample size sufficiently large relative to the number of parameters, so that the use of AICc would provide no substantial benefit over traditional AIC. Indeed, AICc values deviated only slightly from AIC values for our models. Another information criterion, BIC, tends to penalize models with extra parameters more strongly than AIC, thus often favoring simpler models (Burnham and Anderson, 2002; Weakliem, 1999). BIC has a number of other limitations (see Weakliem (1999)), so we chose to base our inferences only on AIC values. Nonetheless, reporting and comparing multiple information criteria may provide additional information on the robustness of model selection.

When interpreting the results of this study, it is important to consider the fact that although the models fitted here represent biological processes, we cannot infer exact evolutionary processes purely from the best-fitting model. There are many more possible ways traits can evolve, and these models are merely mathematical approximations of these processes (Beaulieu et al., 2012). The fact that an OU model fits the data better than other models does not directly prove stabilizing selection, even though stabilizing selection can be modelled as an OU process (Beaulieu et al., 2012). However, fitting a wider variety of models and comparing their fit could provide us with more information on the nature of a trait's evolution. Some examples of models that could be tested include White Noise (*e.g.*, Meireles et al. (2020); Vandelook et al. (2018); Mitchell et al. (2018)) or Late-Burst (Blomberg et al., 2003) models. There are also a number of OU models allowing for distinct directions or models of evolution in different branches of the phylogeny (*e.g.*, Onstein et al. (2016); Vieu et al. (2021)). Although our fitting of OUM models provides an initial exploration of these more complex models, we still assumed equal parameter values for α and σ^2 across dryness categories (Table 3.4). Future studies should further explore the abundance of more complex models that are available, allowing these parameters to vary between groups. It would also be useful to test for differential evolution not only as a function of dryness, but between different clades, regions, altitudes, soil nutrient levels or any other ecological characteristic that might be of interest.

4.4 Conclusions and implications for crop improvement

The conclusions of this study provide potential research paths for crop improvement programs. The traits considered here play key roles in regulating water use, and are therefore important potential targets for improving WUE (Hetherington and Woodward, 2003; Dubberstein et al., 2021). We show that there is substantial phenotypic variation in the six leaf traits studied here, and that different leaf traits are integrated with each other throughout the *Coffea* genus. There is a clear modularity among stomatal traits, with a clear trade-off between stomatal density and stomatal size. This trade-off might be leveraged in crop amelioration in order to adapt coffee production to a broader range of environments. However, this axis was not clearly correlated with climate, indicating that different combinations of stomatal size and density can perform similarly well across environments. Species with larger leaves have increased stomatal densities and possibly also larger stomata, indicating that the trade-off is largely due to physical restrictions: when leaf size increases, both size and density of stomata can increase to allow for higher gas exchange.

Therefore, a more obvious target for selection is leaf area. We show that small-leaved *Coffea* species have evolved in dryer habitats, and larger leaves are found in less arid areas closer to the equator. This is a promising target for future research, considering that the evolutionary models presented here indicate an adaptive significance for leaf area in response to habitat dryness. However, single-trait approaches in crop improvement are unlikely to succeed in improving crop WUE, and an integrative approach is likely to have better results (Flexas, 2016). Our results also suggest a possible adaptive nature of higher leaf area in areas with more temperature seasonality, with stomatal width and SLA also showing possible adaptive value in the face of strong seasonality. However, leaf area did not respond to temperature *per se*, whereas SLA did show possible adaptive value across temperature gradients. Leaf area and SLA thus appear to independently influence different aspects of plant performance. Crop improvement endeavors to improve tolerance to temperature seasonality should focus on WIOI species, which seem to endure more seasonal temperature fluctuations. Continental African species, on the other hand, appear to employ a "faster" ecological strategy with a quicker return on investments in leaf mass, which may be linked to higher average temperatures on the continent.

Throughout the genus, evolutionary divergence is constrained by strong stabilizing selection, and species are capable of adapting rapidly to new environments (as exemplified by *C. macrocarpa* and *C. mauritiana*). Historically, the genus appears to have evolved, on average, towards higher densities of smaller stomata and higher degrees of leaf investment. Further study is required to confirm the relationships presented here, and to assess the practical viability of crop improvement using these traits in coffee wild relatives. The findings presented here contribute to our understanding of leaf trait variation in *Coffea*, and the adaptive significance of these traits.

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Appendices

Appendix A

Risk assessment

Because of the largely computer-based nature of the research performed in this study, potential risks to human health were minimal. No laboratory work was involved in the conducted research, so laboratory risks involving chemicals or large machinery were not present. When sampling leaves from Meise Botanic Garden to account for leaf shrinkage, care was taken to avoid sampling sick plants or plants that might not survive the sampling. Leaves were removed carefully with scissors, which were handled with caution to avoid injury to the fingers or hands.

Some minor risks during analyses and manuscript writing should also be taken into account. Extended periods of computer work can lead to eye strain and postural issues with the neck and back. Frequent breaks were thus given a high priority, making sure to move around and go outside regularly. Monitor brightness was also adjusted to minimize discomfort to the eyes.

Appendix B

Full annotated phylogeny of the included species

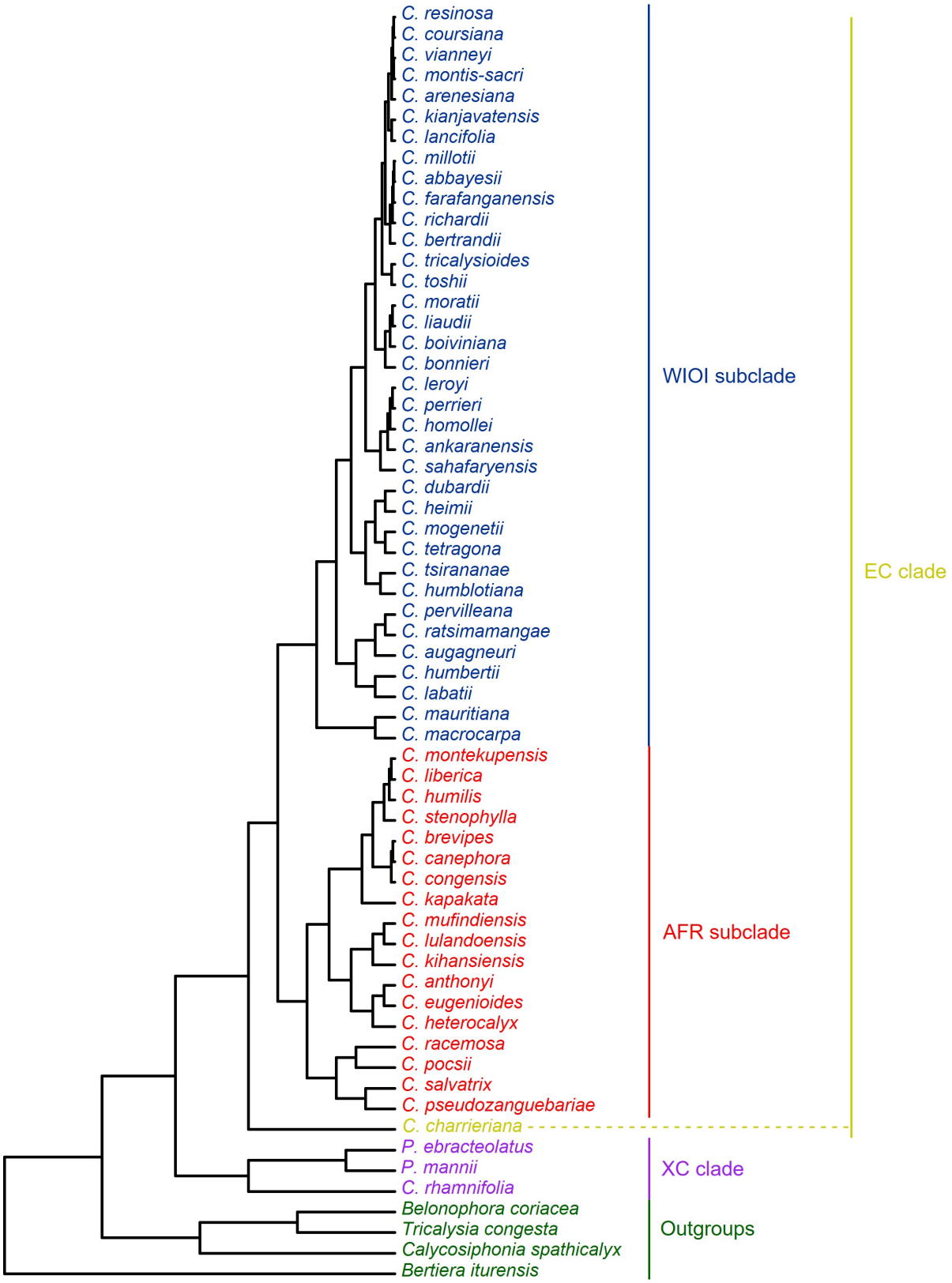

Figure B.1: Full phylogenetic relationships of the species included in this study, based on the phylogeny of Hamon et al. (2017).

Appendix C

Pagel's lambda likelihood profiles

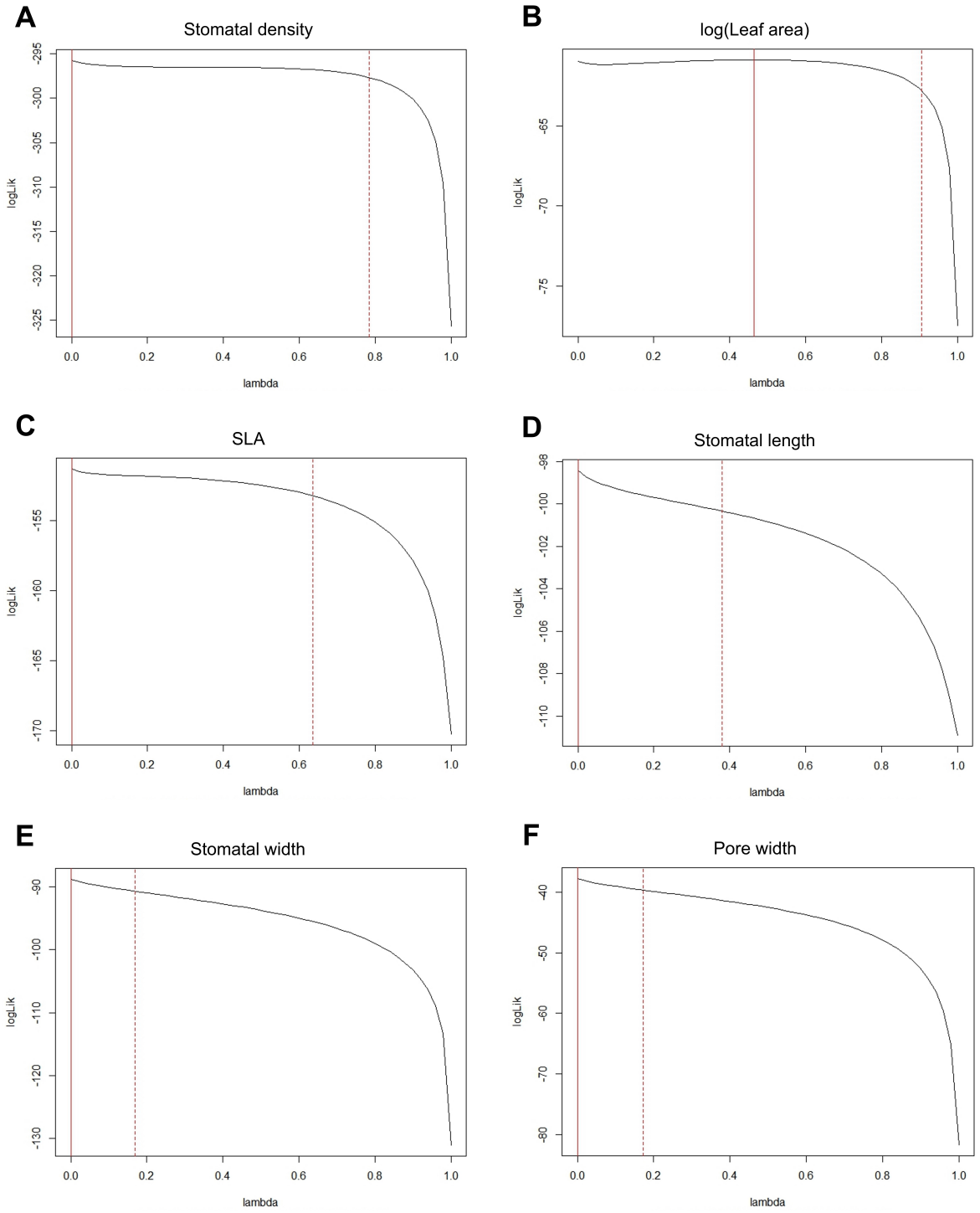

Figure C.1: Likelihood profiles for the estimates of Pagel's λ obtained simultaneously with the pGLS models. Solid red lines indicate the maximum likelihood estimate for λ. Dotted red lines indicate 95% confidence intervals.

Appendix D

Box plot of climate PC2 values per dryness category

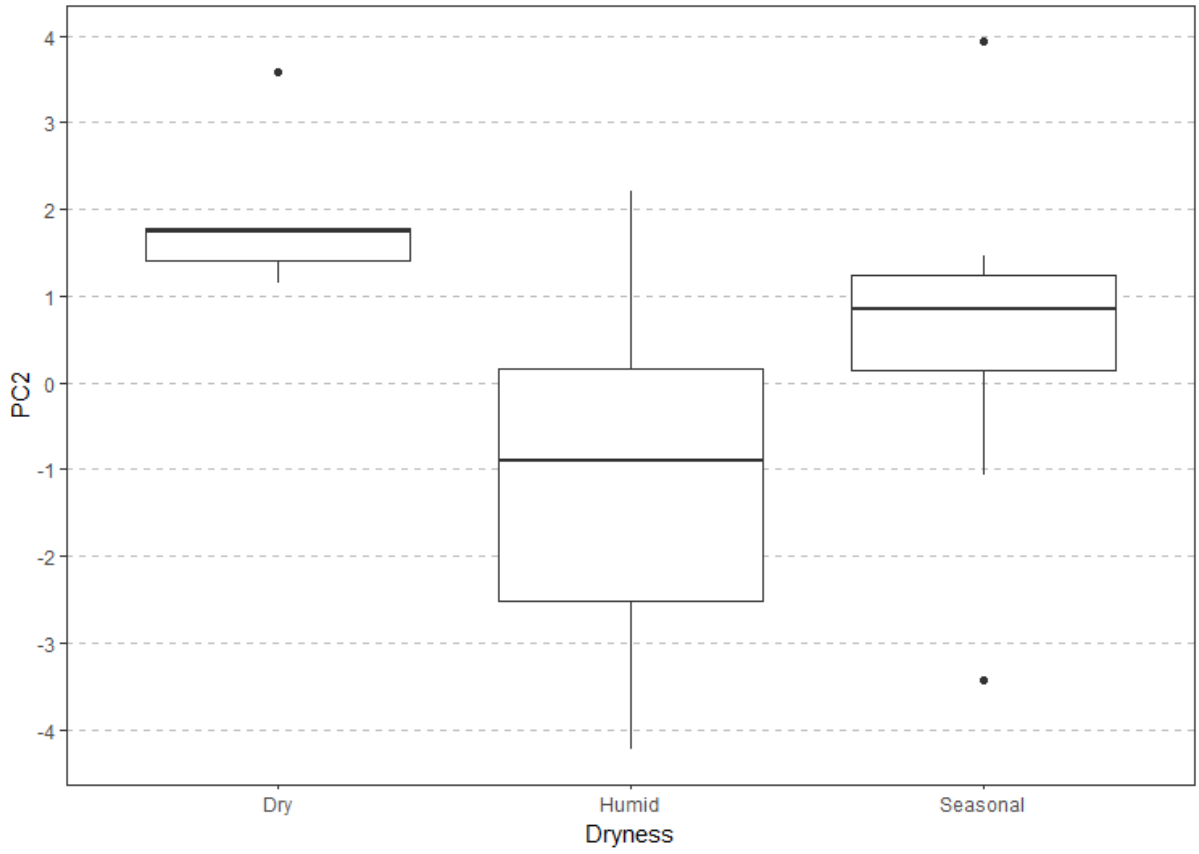

Figure D.1: Box plot showing the climate PC2 value by dryness category for all included species in the data set. Outgroup species were included in the data for this figure.

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