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THE EVOLUTION AND ECOLOGY OF DEFENSIVE TRAITS IN ANTS

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BY

BENJAMIN DAVID BLANCHARD

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Dedicated to Daddy, Mama, Belly-Buddy, Evvy-Face, and The Whats

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Acknowledgments

As I write this section of my dissertation – the final unfinished component of the document – a global pandemic spreads around the globe, between cities, and within our communities. It was always inevitable that the completion of this six-year project would compel me to pause and reflect on the constellation of marvelous people without whom I would be in a spiritually and intellectually worsened state. The uncertainties of this current time only highlight what I've always known is true: I love the frass out of all of you!

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“While an ant was wandering under the shade of the tree of Phæton, a drop of amber enveloped the tiny insect; thus she, who in life was disregarded, became precious by death.”

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Did I get everyone? I hope so, because I'm not doing *this* again!

Introduction

Across the tree of life, species diversity and trait divergence abounds. Ants are no exception. Within this single family of Insecta, evolution has produced a stunning array of species boasting behaviors and morphologies ranging from obligate mutualisms with Hemipterans and plants (Stadler et al., 2005, Calixto et al., 2018), high-speed trap-jaws exhibiting some of the fastest movements of any animal (Larabee & Suarez, 2014), flattened heads serving as shields to defend against invaders (Powell, 2016), massive nomadic colonial migratory behavior (Wilson, 1958), fungus farming (Mehdiabadi & Schultz, 2010), ovipositors transformed into a sting or acidopore capable of delivering a number of different venoms (Blum, 1992), and a host of other traits whose simple description alone could easily fill the space of an entire dissertation. Faced with such a remarkable display of variation, my doctoral work is driven by the question: What drives the evolution of variable defensive traits in ants, and what constraining forces might limit morphological disparity? Across the three chapters of my dissertation, I approach this question from both macroevolutionary and ecological perspectives.

Top-down processes, like predator-prey interactions, have seen little attention in myrmecological research (Cerdá et al., 2013), and defensive trait-based theories are thus largely overlooked in the literature. In my first chapter, I investigate the potential role of evolutionary trade-offs (Stearns, 1989; Tilman, 2011) in constraining the evolution of morphological defensive traits in ants and assess the impact of these traits on diversification. While there have been some recent studies on morphological evolution in ants in single genera or in geographically restricted communities (Pie and Traniello 2007, Moreau 2008, Sarnat and Moreau 2011, Blaimer et al. 2015, Holley et al. 2016), little work has integrated phylogenetic and morphological data to address trait-based diversification and evolutionary processes in ants on a broad, family-wide scale. This work is therefore novel in its phylogenetic breadth and also provides the evolutionary context and motivation for the following two dissertation chapters. I found evidence for an evolutionary trade-off between the chemical stinger and a suite of other defensive traits including cuticular spines, polymorphism, large eye size, and large colony size. Furthermore, cuticular spines, along with a couple other traits, are associated with significantly higher rates of diversification. Taken together, my first chapter suggests that defensive traits meaningfully influence macroevolutionary patterns in ants and warrant serious consideration.

To establish potential mechanisms driving evolutionary and ecological success, it is necessary to conduct ecological trials that assess trait functions and possible costs associated with a trait. Thus, for my second chapter, I target the hyperdiverse spiny ant genus *Polyrhachis*, which exhibits a wide range of spine traits from no spines to multiple large and curved thorn-like projections reaching lengths matching the length of the entire thorax. Through a number of lab trials with live colonies collected in and near the Xishuangbanna Tropical Botanical Garden in southern Yunnan, China, I show that species with longer spines either outperform or do not differ from species with shorter spines in resource discovery rate, foraging effort, competitive ability, and anti-predator defense. I therefore do not find evidence of an ecological trade-off (Stearns, 1989; Kneitel & Chase, 2004), and instead find that defensive spines may partially explain the ecological success of ants. Although challenging, future ecological research in ants should attempt a more multivariate framework as has proven productive in plant systems (Agrawal & Fishbein, 2006).

Building upon my first two chapters, I sought to assess the consequences of apparent ecological benefits of spines for species diversification in *Polyrhachis*. After utilizing a phylogenomic approach through sequencing of ultraconserved elements (Faircloth et al., 2012) to infer a well-supported phylogeny that doubles the number of described taxa sampled relative to the previous molecular study (Mezger & Moreau, 2016), I compiled a trait database including spine length, body size, and geographic range size data. Through macroevolutionary analyses using these data, I find that there is no association between spines and diversification in this group, but do find a positive association between spine length and geographic range size. These results provide some support for one expectation of the escape-and-radiate hypothesis - namely expanded niche range following escape from enemies driven by the evolution of a defensive trait (Ehrlich & Raven, 1964) - but do not support elevated diversification rates as expected from my first chapter and found in groups like plants (Weber & Agrawal, 2014) and poison frogs (Arbuckle & Speed, 2015).

Overall, the three chapters of this dissertation establish defensive traits, and especially spines, as important factors in ant evolution. While the first two chapters support spines as drivers of evolutionary and ecological success, the third chapter highlights potential complexities of this association while additionally suggesting that spines promote increased geographic range size as well. Future work on defensive traits in ants is likely to produce informative tests of trait-based diversification and the impact of functional traits on ant ecological interactions.

1 Defensive traits exhibit an evolutionary trade-off and drive diversification in ants

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1.1 Abstract

Evolutionary biologists have long predicted that evolutionary trade-offs among traits should constrain morphological divergence and species diversification. However, this prediction has yet to be tested in a broad evolutionary context in many diverse clades, including ants. Here, we reconstruct an expanded ant phylogeny representing 82% of ant genera, compile a new family-wide trait database, and conduct various trait-based analyses to show that defensive traits in ants do exhibit an evolutionary tradeoff. In particular, the use of a functional sting negatively correlates with a suite of other defensive traits including spines, large eye size, and large colony size.

Furthermore, we find that several of the defensive traits that trade off with a sting are also positively correlated with each other and drive increased diversification, further suggesting that these traits form a defensive suite. Our results support the hypothesis that trade-offs in defensive traits significantly constrain trait evolution and influence species diversification in ants.

1.2 Introduction

All species experience constraints arising from developmental, functional, and energetic limitations. These limitations have featured prominently in the development of ecological and evolutionary theories since Darwin (Darwin, 1859), and include optimal foraging theory (Krebs, 1978), key innovation theory (Crother et al., 2007), and life-history theory (Stearns, 1989; Tilman, 2011). One concept shared by these theories is the importance of tradeoffs between different investment strategies. Ecological theories on tradeoffs predict differential energy investment in different traits based on resource limitations or conflicting environmental demands (e.g., Werner & Anholt, 1993). In contrast, evolutionary trade-off theory posits that energy investment in a given trait in response to some selective force limits investment in other traits that serve similar functions. Therefore, such traits should be negatively associated with each other across species over evolutionary time (Stearns, 1989; Arnold, 1992; Mooney et al., 2010; Tilman, 2011; Ferenci, 2016).

Trait trade-offs influence various evolutionary processes, including patterns of morphological divergence (DeWitt et al., 2000; Svanbäck & Eklöv, 2003; Muchhala, 2007; Holzman et al., 2012; Heinen-Kay et al., 2015) and rates of diversification (Schluter, 1995; Holzman et al., 2012). For example, Muchhala (2007) found that increased specialization of floral morphology in *Burmeistera* is mediated by an adaptive trade-off arising from differential efficiency of bat and hummingbird pollination.

Holzman et al. (2012) showed that biomechanical trade-offs in the feeding morphology of fishes promote elevated rates of evolution in the traits contributing to the trade-off. Trade-offs could ultimately limit rates of speciation or morphological evolution by restricting species to particular regions of morphospace or ecological niches (Shoval et al., 2012), as seen in guppies (Ghalambor et al., 2004) and bacteria (Ferenci, 2016). However, others have suggested that trade-offs may promote diversification in some cases (Schluter, 1995; de Vos et al., 2015). For example, Herrel et al. (2009) find evidence for a trade-off between bite force and jaw movement velocity in Darwin's finches, and suggest that this trade-off may lead to differences in the evolution of song production and, as a consequence, drive reproductive isolation and subsequent speciation.

In practice, it can be difficult to assess the role of trait tradeoffs over evolutionary timescales. Determining the ecological function of focal traits is important, as a negative correlation between traits may not be the a priori expectation for sets of traits that are not functionally redundant (Agrawal, 2011). Furthermore, the variability and diversity of processes acting on morphological and species diversification may influence evolutionary dynamics, leading to unexpected patterns (Bennett & Lenski, 2007). However, despite these challenges, evidence for an evolutionary trade-off can be assessed in a system with labile traits of known or likely functions and a fairly complete and sizable phylogeny (Maddison & FitzJohn, 2015).

Over the past several decades, there has been much interest in ants as a group of insects that are highly influential in many terrestrial ecosystems (Hölldobler & Wilson, 1990; Lach et al., 2010). In particular, recent work has significantly improved our understanding of phylogenetic relationships between ant species, genera, and subfamilies (Brady et al., 2006; Moreau et al., 2006; Lapolla et al., 2010; Ward et al., 2010; Moreau & Bell, 2013; Schmidt, 2013; Blaimer et al., 2015a; Brady et al., 2015; Ward et al., 2015), providing a crucial foundation for understanding evolutionary processes. Furthermore, a growing body of research has focused on relationships between morphological traits and ecological niche in ants. Such eco-morphological relationships include eye size and diet (Weiser & Kaspari, 2006), caste specialization and prey type (Powell & Franks, 2006), and morphological specialization and latitude (Silva & Brandão, 2014). Additionally, a few studies on single genera or geographically restricted communities have correlated aspects of morphological evolution with some specific evolutionary or ecological process such as ecological release or niche adaptation (Wilson, 1959; Pie & Traniello, 2007; Moreau, 2008; Sarnat & Moreau, 2011; Blaimer et al., 2015b; Price et al., 2016). However, to date, relatively little work has integrated phylogenetic and eco-morphogical data to address trait-based diversification and evolutionary processes in ants, especially on a broad, family-wide scale.

Defensive traits are compelling candidate drivers of evolution across many taxa, including in ants (Hunt, 1983), and may be strongly selected as an anti-predator defense. Predation has been invoked as an important selective force influencing clade diversification and morphological divergence across many taxa, including plants (Agrawal, 2011; Weber & Agrawal, 2014; Endara et al., 2015), amphibians (Arbuckle & Speed, 2015), dragonflies (Mikolajewski et al., 2010), and beetles (Ge et al., 2011). In ants, traits thought to confer defensive benefits to a colony are remarkably varied across genera, including stings, spines, chemical defenses, recruitment and deployment of a soldier caste, and cryptic foraging strategies (Hunt, 1983; Lach et al., 2010). These key traits are likely costly and under strong selection pressure, and should therefore exhibit some trade-off in energy investment. The diversity of defensive morphologies and life-history strategies suggests there may be an evolutionary trade-off among some of these traits. This hypothesis is corroborated by observational evidence of defensive trait trade-offs across subfamilies and genera (Moreau, pers. obs.), but the role of trait trade-offs in constraining or otherwise influencing evolution in ants has not yet been rigorously tested. In fact, most general patterns relating to ant morphological defense have not been investigated (Lach et al., 2010).

We reconstruct an expansive, genus-level phylogeny of ants that includes 268 described genera (82% of all described ant genera), compile a genus level database for eight morphological and ecological traits, and conduct trait-based diversification and phylogenetic correlation analyses to address whether defensive traits exhibit evolutionary trade-offs in ants. These data and analyses additionally allow us to investigate the evolutionary lability of multiple defensive traits and the impact of these traits on rates of diversification. Although functional traits are likely to play a significant role in ant evolution, the influence of these traits on patterns of diversification have rarely been investigated, and the processes constraining the evolution of these traits are still unknown. This study will therefore provide a foundation for future work on trait-based evolutionary processes in ants.

1.3 Materials and methods

1.3.1 Molecular data

We inferred a genus-level molecular phylogeny of the ants using sequences from previously published studies (Lapolla et al., 2010; Ward et al., 2010; Moreau & Bell, 2013; Schmidt, 2013; Brady et al., 2014; Ward et al., 2015) and 19 additional sequences from GenBank (Supplementary Table S1.1). The sequences retrieved from Moreau and Bell (2013) are derived from three prior studies (Brady et al., 2006; Moreau et al., 2006; and Rabeling et al., 2008). From these sources, sequences were available for 648 ant species covering 268 described genera, as well as 18 outgroup taxa, spanning nine nuclear and mitochondrial genetic markers. For each species, all available genes were included even if derived from different individuals. In total, 45.2% of species had data for all nine genes, and 96.1% of species had data for four or more genes. Sequences were aligned using MUSCLE in R (Edgar, 2004; R Core Team, 2015). The alignment was then inspected visually, and ambiguously aligned sites were excluded manually in Mesquite v3.02 (Maddison & Maddison, 2015). The final aligned data matrix was deposited in TreeBASE (URL: http://www.purl.org/phylo/treebase/phylows/study/TB2:S19313; http://www.treebase.org).

1.3.2 Phylogenetic inference

An initial tree with 666 terminals was inferred under a maximum likelihood framework in RAxML v8 (Stamatakis, 2014) on the CIPRES computing cluster (Miller et al., 2010). The concatenated matrix of all nine genes was partitioned so that the GTR+Γ model of evolution for each gene was independent of each other. Support for inferred relationshipswas determinedwith bootstrapping, with the program automatically halting bootstrapping after a sufficient number of replicates, and the maximum likelihood tree was then pruned to include only one species per genus, to control for uneven genus sampling across the tree. Eighteen genera included in our study that are currently recognized in the literature are known to actually represent a polyphyletic grouping of species, but have yet to be formally revised. When this was the case, we either chose one species as the representative for the genus or retained multiple lineages with distinct identifying names (see Supplementary Table S1.2 for a more detailed description of our selection process following the recent taxonomic literature). When we retained multiple tips for a genus (only four of our 268 described ant genera), we used the same trait states for the genus for all tips and evenly divided the number of species in the genus by the number of tips in our tree. In addition to 16 outgroup taxa from related Hymenopteran clades, the pruned genus-level tree includes 275 ingroup ant species from 268 described ant genera (82.0% of all extant ant genera), an increase of 105 genera compared to the most recent family-wide phylogenetic study (Moreau & Bell, 2013).We will subsequently refer to the ingroup and outgroup taxa as “genera,” for a total of 291 genera. Using these 291 genera, we then inferred another tree in RAxML using the same settings described above, and this genus-tree was used as a starting tree for divergence time estimation.

1.3.3 Divergence dating

Divergence time estimation using Bayesian inference was conducted in BEAST v1.7 (Drummond et al., 2012) on the CIPRES computing cluster. The inferred maximum likelihood tree (291 genera) was used as the starting tree in BEAST. A total of 73 fossils, including 69 ingroup fossil taxa, were used to calibrate node age priors under a lognormal prior distribution with an offset corresponding to the minimum fossil ages (Supplementary Table S1.3). A GTR + Γ model of sequence evolution was used for our gene-partitioned dataset, with parameters across partitions unlinked. All settings were implemented using BEAUti v1.7 (Drummond et al., 2012), which produces the XML file formatted for BEAST. After initial test runs, the MCMC length was set to 200 million generations (sampled every 10,000 generations), the upper bound of the ucld.mean parameter to 0.1, and the mean of ucld.stdev to 0.6, with all other settings left at the default. We then used Tracer v1.6 (Rambaut et al. 2014) with a burn-in of 10% to assess effective sample sizes for each parameter and ensure adequate convergence, and Tree Annotator v1.8.2 (Drummond et al., 2012) with 10% burn-in and a posterior probability limit of 50% to generate the dated maximum clade credibility tree. This MCC tree was deposited in TreeBASE (URL: http://www.purl.org/phylo/ treebase/phylows/study/TB2:S19313; http://www.treebase.org).

1.3.4 Morphological and ecological data

Trait data were compiled from information in the primary literature and online databases (Supplementary Table S1.4; database deposited in the Dryad Digital Repository, URL: http://dx.doi.org/ 10.5061/dryad.st6sc). We collected data for five traits with a purported defensive function: cuticular spines (“spinescence” or “spines”), large eye size, worker polymorphism, unmodified venom-delivering sting (“sting”), and large colony size. Although each of these traits may have additional functions (e.g., foraging efficiency), they likely also serve a defensive role in many or most species in which they occur. Spines and the sting are both generally considered a direct defense against vertebrates or invertebrates (Lach et al., 2010; Schmidt, 2014). Large eyes likely confer increased visual acuity allowing individuals to better avoid attackers (Cronin et al., 2014). Worker polymorphism, and in particular the recruitment of a soldier caste, is often used as a defense against arthropods (Lamon & Topoff, 1981; Hunt, 1983; Powell, 2008; Kamhi et al., 2015). Large colony sizes may buffer against individual worker loss (Kaspari & Vargo, 1995) or allow a colony to better defend itself against attackers (Adams, 1990). In addition to these five traits, we collected data on three ecological niche traits: diet (herbivore, omnivore, or predator), nesting location (on the ground or arboreally), and foraging location (on the ground or arboreally). Historically, morphological and ecological data collection for ants has been heavily biased toward the worker caste, so we restricted our database to genera for which the worker caste is known. Traits were scored for each ant genus, and a genus was considered polymorphic for a trait if at least 10%

of the species in the genus had an alternative trait state. In some cases, there was no information on a trait for a genus in the primary literature or online database records, but we assigned a state for the trait based on the likelihood of the trait state given background expectations. For example, a genus was considered to be monomorphic in the absence of documented evidence of polymorphism. These cases are clearly denoted in Supplementary Table S1.4. As several analyses used in this study require traits to be categorical or binary, trait states were binned into discrete categories, and in some cases alternative binning schemes were used to test different hypotheses (see Supplementary Table S1.5 for a discussion of trait state binning). For all analyses, polymorphic scores for a trait in a given genus were converted to the nonzero number (e.g., a score of “0&1” was converted to “1”).

1.3.5 Ancestral state reconstruction

Ancestral state reconstructions were estimated for each trait at each node of the maximum clade credibility tree using the make.simmap and describe.simmap functions in phytools v0.5-54 (Revell, 2012), which utilize a stochastic character mapping approach (Huelsenbeck et al., 2003; Bollback, 2006). The model of trait evolution used for each trait was determined by comparing the log likelihoods of three possible models: “Equal rates” (ER), “Symmetrical rates” (SYM), and “All rates different” (ARD), referring to rates of trait state changes for each trait. The model with the highest log-likelihood was chosen if the difference from each simpler model was greater than 2, which is a standard cut-off for this method (Paradis et al., 2004). We set the optimization method to “optim,” and all other settings were left at the default. For each trait, we then ran 500 simulations, and the percentage of simulations for which each trait state was inferred at each node was then plotted as a pie chart on our maximum clade credibility tree. We also assessed the evolutionary lability of each trait using this approach, which returns the inferred number and type of changes occurring across the tree for each trait.

1.3.6 Shifts in diversification rates

We used MEDUSA (Alfaro et al., 2009) to identify diversification rate shifts across the maximum clade credibility tree. MEDUSA uses a stepwise Akaike information criterion (AIC) method as implemented in geiger v2 (Harmon et al., 2008; Pennell et al., 2014). This method first assigns a background rate of diversification to an entire tree and calculates the likelihood for this singe rate regime under a birth-death (BD) or pure-birth (Yule) model. The program then searches for the most likely location for a rate shift based on AIC score. This more parameter-rich model is then compared to the model with only the background rate and is accepted if it results in a decrease in AIC score above a user-defined threshold (default = 4 AIC units). This process continues for models with an increasing number of rate shifts until there is no longer a decrease in AIC score greater than the user-defined threshold. To account for incomplete sampling, the number of species represented by each tip (i.e., genus in our case) was included (Bolton, 2014; Supplementary Table S1.4). We allowed the program to determine the best-fitting model of evolution for each shift (BD or Yule), and all other settings were left at the default. We then used Fisher's exact test (two-tailed) to investigate associations between rate shifts (increases and decreases) and defensive traits based on node states inferred from the ancestral state reconstruction. Preliminary analyses suggested that trait shifts in trait states very rarely occurred at the same nodes as rate shifts, so we only investigated node trait states, given that shifts in diversification due to a trait can also occur on a phylogeny downstream of the trait's origin (e.g., McKenna et al., 2009). However, although the rate shifts are distributed throughout our phylogeny, this method may suffer from pseudoreplication. We therefore consider this test to be a supplement to our core trait-based diversification analysis (below).

1.3.7 Sister clade analysis

Assessing the influence of traits on diversification across clades is a classically difficult problem (Jablonski, 2005; Vamosi & Vamosi, 2011; Ng & Smith, 2014; Maddison & FitzJohn, 2015). New methods involving "state-dependent speciation and extinction" (SSE) models, including the BiSSE model and several derivatives, appeared to offer a promising solution to known issues with earlier methods (Ng & Smith, 2014), such as the difficulty of inferring ancestral states for traits that also drive diversification (Maddison, 2006). However, recent analyses have shown that the SSE suite of models frequently suffer from high rates of model inadequacy, sometimes resulting in the null model being rejected in 100% of tested cases, and thus often fail to properly account for phylogenetic relationships between species and clades (Maddison & FitzJohn, 2015; Rabosky & Goldberg, 2015). Preliminary analyses using our data and maximum clade credibility tree suggested that our tree is prone to the sort of model inadequacy highlighted by Rabosky and Goldberg (2015); therefore, we decided to instead use sister clade analysis. This more conservative method, while known to have somewhat low statistical power (Ng & Smith, 2014), also explicitly ensures phylogenetic independence of included comparisons and is thus less prone to the artificially high rates of null model rejection seen in the SSE models (Maddison & FitzJohn, 2015; Rabosky, pers. comm.). The results from our ancestral state reconstructions suggested that there were enough sister clades with differing states for all the traits included in this study to justify using sister clade analysis. Furthermore, most traits show numerous state transitions throughout the phylogeny, likely minimizing issues related to large imbalances in trait states in trait-based diversification analyses (Maddison, 2006). Thus, we use sister clade analysis as a fairly conservative estimate of associations between traits and clade diversification.

We conducted sister clade comparisons based on plots of ancestral state reconstructions for our five morphological and three ecological traits. For each trait, sister clades were compared if the two clades differed in trait states and every genus in each clade shared a state for the trait. There were at least 10 and typically 20 or more such sister clades for our traits. We then implemented a model-based sister clade analysis that incorporates species numbers and time since divergence for each sister clade (Paradis, 2011). This analysis uses a likelihood ratio test to compare the fit of two models of diversification—a null model and an alternative model where a trait increases or decrease rates of diversification. This method has been shown to be more powerful than previous techniques, and is implemented using the function richness.yule.test in the R package ape (Paradis et al., 2004). For each sister clade, we used the species numbers for each genus in our dataset (Supplementary Table S1.4) and the dates of divergence inferred from our maximum clade credibility tree.

1.3.8 Analysis of trait correlation

To assess the evidence for an evolutionary trade-off between defensive traits, we performed a phylogenetic maximum likelihood correlation test based on Pagel's method (Pagel, 1994; Pagel & Meade, 2013; Beaulieu & Oliver, 2015). The phylogenetic correlation test was conducted using corDISC in corHMM v1.16 (Beaulieu et al., 2015), which tests hypotheses of correlated evolution between two binary traits. This test compares the log likelihood of a four-parameter model, in which each trait evolves independently of the other trait, to the log-likelihood of an eight parameter model that incorporates different transitions rates for one trait based on the state of a second trait (Fig. 1.1). The dependent (i.e., correlated) model is favored if the log-likelihood of the dependent model is significantly higher than that of the independent (i.e., uncorrelated) model, as determined by a likelihood ratio test (LRT) with four degrees of freedom.

In cases where the dependent model was favored, we tested additional hypotheses to determine which specific transition rates were affected by the associated trait. These hypotheses were tested by constraining certain pairs of parameters to be equal and comparing the constrained model to the full eight-parameter model (henceforth called the "parameter restriction test"). For example, to test for evidence of a trade-off between eye size and a sting, the transition rate from small eyes to large eyes in the absence of a sting was constrained to be equal to the transition rate from small eyes to large eyes in the presence of a sting (Fig. 1.1B). If the log-likelihood of this constrained, seven-parameter model is significantly lower than the full eight-parameter model (ascertained using a LRT with one degree of freedom), and the transition rate from small eyes to large eyes is higher in the absence of a sting, then there is support for a negative correlation (trade-off) between eye size and a sting. But if the rate from small eyes to large eyes is higher in the presence of a sting, then there is support for a positive correlation between the two traits. Thus, when there was evidence that two traits were correlated, the following were tested using the parameter restriction test (Fig. 1.1B):

1. Difference in transition rate from absence to presence of Trait 1 based on state of Trait 2.
2. Difference in transition rate from absence to presence of Trait 2 based on state of Trait 1.
3. 3. Difference in transition rate from presence to absence of Trait 1 based on state of Trait 2.

4. 4. Difference in transition rate from presence to absence of Trait 2 based on state of Trait 1.

These tests of correlation were conducted using the maximum clade credibility (MCC) tree for all possible pairwise comparisons between all traits, including for each different binning schemes for the ecological traits (diet, nesting, and foraging; see Supplementary Table S1.5 for binning schemes).

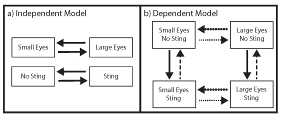

Figure 1.1: Example of models compared in corHMM to test for correlations between binary traits. The log-likelihood of the four parameter, independent model (A) was compared to the eight-parameter, dependent model (B). If the eight-parameter model was significantly more likely (i.e., traits are correlated), an additional test (parameter restriction test) was conducted to determine the transition rate differences that significantly increase model likelihood. Four parameter restriction tests were conducted, with one pair of traits constrained to be equal in each test (B, indicated by line type). The likelihood of each of these seven-parameter models was compared to the full eight-parameter model, and parameter values are considered to be significantly different if there is a significant decrease in likelihood when the parameters are constrained.

1.4 Results

1.4.1 Phylogenetic inference

The topology of the full species ML tree (666 tips, Supplementary Fig. A.1) and the maximum clade credibility (MCC) Bayesian genus-tree (291 tips, Supplementary Fig. A.2) are similar, and both are largely consistent with previous studies (LaPolla et al., 2010; Ward et al., 2010; Moreau & Bell, 2013; Schmidt, 2013; Brady et al., 2014; Ward et al., 2015). The posterior support values for most nodes in our tree are modest to strong (0.75–1.0), although a portion of the Ponerinae clade includes a relatively high number of nodes with low support (Supplementary Fig. A.2). The present tree differs in the relationships between subfamilies from the most recent family-wide phylogenetic study (Moreau & Bell, 2013) only in that Martialinae is inferred here to be the sister group to all other extant ants (with low support, PP = 0.41) as found in Rabeling et al. (2008) and Schmidt (2013), whereas Moreau et al. (2006), Kück et al. (2011), and Moreau and Bell (2013) found Leptanillinae to be sister. All other subfamily relationships match Moreau and Bell (2013). Major phylogenetic relationships at the tribe level are also consistent with previous subfamily analyses (Ward et al., 2010; Schmidt, 2013; Ward et al., 2015), with a couple exceptions in Dorylinae (Brady et al., 2014). In this study, the Doryline genera *Amyrmex* and *Leptanilloides* were found within the "army ant" clade, whereas in Brady et al. (2014) these genera form their own clade. Furthermore, Brady et al. (2014) found *Vicinopone* as the basal-most Doryline genus, while in this study *Tanipone* takes this position.

1.4.2 Divergence dating

The divergence-dated chronogram, generated from the BEAST analysis using the ML topology enforced as the starting tree, recovered a mean root age of 176.1 Mya [95% highest posterior density (HPD) 147.3 to 214.4 Mya] for crown-group ants (Supplementary Fig. A.2, Supplementary Table S1.6). This tree includes 275 in-group genera, with 82% of extant ant genera represented. The dating of most major clades, including the largest subfamilies Myrmicinae, Formicinae, Dolichoderinae, and Ponerinae, is congruent with previous studies (Supplementary Table S1.6). A notable exception to this is the inferred age of crown-group ants, which in this study is inferred to be about 25 million years earlier than the most recent family-wide analysis (Moreau & Bell, 2013; Supplementary Table S1.6). Although the inferred age of crown Formicidae and two subfamilies in our MCC tree are somewhat older than most recent studies, the majority of internal nodes are consistent with at least one of these studies, so we accepted this tree as adequate for subsequent analyses. Our MCC tree includes four genera without a known worker caste, which we excluded using the drop.tip function in ape (Paradis et al., 2004), resulting in a tree used for all downstream analyses that includes 271 ant genera (Fig. 1.2).

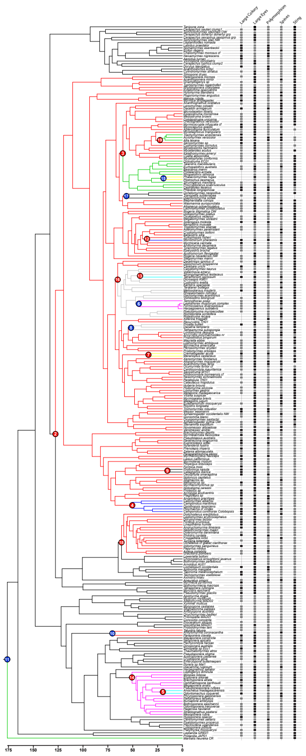

Figure 1.2: Dated maximum clade credibility (MCC) tree with trait states and MEDUSA rate shifts mapped. Outgroups are excluded. Scale bar is in millions of years before present. Trait states for each tip are denoted using a black square (trait present), a gray square (trait absent), or no square (no data). Nodes at which rate shifts occur are denoted by colored branches and labeled with a circle (red = rate increase, blue = rate decrease). The background rate begins at the root node.

1.4.3 Morphological and ecological data

A dataset for eight scored morphological and ecological traits was generated for all 313 genera for which the worker caste is known, out of the total 326 extant ant genera (Supplementary Table S1.4). Some data is missing for traits in some genera, but coverage is high across the 313 genera. Traits with missing data are sting (95.8% total coverage), diet (83.7%), nesting location (98.4%), foraging location (97.4%), and colony size (69.3%). Among the 271 ant genera in our tree, coverage is even higher for traits with missing data, which include sting (96.7%), diet (86.7%), foraging location (99.6%), and colony size (76.8%). For subsequent analyses involving a trait with incomplete taxonomic coverage, genera with missing data for that trait were pruned from the phylogeny using the drop.tip function in ape (Paradis et al., 2004), unless the analysis could incorporate missing data (e.g., correlation test in corHMM).

1.4.4 Ancestral state reconstruction

Ancestral state reconstructions revealed a range of evolutionary lability across the eight morphological and ecological traits (Supplementary Figs. A.3–A.10). The least labile trait was a functional sting, while the most labile traits included nesting location and colony size (Supplementary Table S1.7). Despite the general lability of colony size across all ants, the evolution of large colony size only occurred once each in several subfamilies (Supplementary Fig. A.3), as seen in the recent analysis of Burchill and Moreau (2016). Among the 14 changes in sting presence across the tree, none were inferred as secondary gains. This directionality of trait state transitions was also found, to a lesser degree, for spines (loss of spines more than twice as likely as gain), and eye size (eye size increase 50% more common than decrease). Furthermore, the majority of shifts in diet were to a predacious or herbivorous diet, with only 14 shifts to omnivory.

Table 1.1: Rate shifts and rate shift parameters inferred using MEDUSA (+) indicates a significant rate increase, and (−) indicates a significant rate decrease. r = net diversification rate ($\lambda - \mu$, where λ = speciation rate, and μ = extinction rate), and ϵ = relative extinction rate ($\epsilon = \mu/\lambda$).

Clades in rate regime	Direction [r]	# in Fig. 1.2	r	ϵ
	Background	N/A	0.0309825	0.91215
Heteroponerinae, Ectatomminae, and large portion of Formicinae and Myrmicinae	+	2	0.0598134	0.74505
Octostruma, Talaridris, Eurhopalothrix, Basiceros, Protalaridris, Rhopalothrix, Strumigenys, Procryptocerus, Cephalotes, and *Pheidole*	+	3	0.1031564	0.88741
Calomyrmex, Camponotus, Camponotus (Colobopsis), Opisthopsis, and *Polyrhachis*	+	4	0.0606868	0.9801
Anochetus and *Odontomachus*	+	5	0.2278795	NA
Crematogaster and *Meranoplus*	+	7	0.1675758	NA
Proformica and *Cataglyphis*	+	9	0.2755019	NA
Monomorium	+	12	0.165836	NA
Tetramorium	+	13	0.1466766	NA
Leptogenys, Myopias, Euponera, Euponera, Brachyponera, Ophthalmopone, Megaponera, Pseudoneoponera, Phrynoponera, Paltothyreus, Buniapone, Bothroponera, Odontoponera, Hagensia, Streblognathus, and *Mesoponera*	+	14	0.0943411	NA
Calyptomyrmex, Vollenhovia, Strongylognathus, Dicroaspis, Cyphoidris, Kartidris, Terataner, Melissotarsus, Rhopalomastix, Gauromyrmex, Vombisidris, Poecilomyrma, Romblonella, and *Rotastruma*	+	16	0.068061	NA
Dolichoderus, Leptomyrmex, Dorymyrmex, Forelius, Linepithema, Anonychomyrma, Nebothriomyrmex, Deleromyrma, Philidris, Froggattella, Turneria, Ochetellus, Iridomyrmex, Papyrius, Azteca, and *Gracilidris*	+	18	0.0833777	NA
Trachymyrmex, Acromyrmex, and *Atta*	+	19	0.2129122	NA
Temnothorax, Leptothorax, Formicoxenus, and *Harpagoxenus*	-	6	0.0018187	0.9996
Proatta, Dacatria, and *Tetheamyrma*	-	8	0.0069088	NA
Tatuidris, Ankylomyrma, and *Paraponera*	-	10	0.0057103	NA
Martialis	-	11	0	NA
Phalacromyrmex and *Pilotrochus*	-	15	0	NA
Ochetomyrmex, Tranopelta, and *Diaphoromyrma*	-	17	0.0169692	NA

1.4.5 Shifts in diversification rate

The MEDUSA analysis of our dated phylogeny detected 18 significant rate shifts in diversification, including 12 rate increases and six rate decreases (Table 1.1). The largest single shift was a rate increase that includes four subfamilies: Heteroponerinae, Ectatomminae, Myrmicinae, and Formicinae (Table 1.1, Fig. 1.2). There were no rate shifts detected in Dorylinae, Pseudomyrmecinae, Amblyoponinae, Aneuretinae, or Proceratiinae (Fig. 1.2).

Based on Fisher's exact test and the ancestral state reconstructions at nodes where rate shifts occurred (Supplementary Table S1.8), there is a trend of marginal significance suggesting that large eye size is associated with rate increases ($P = 0.08$, Table 1.2), as well as a positive but nonsignificant association between large colony size and rate increases ($P = 0.26$, Table 1.2). There was also a nonsignificant positive association between a sting and rate decreases ($P = 0.25$, Table 1.2). Spines and polymorphism were not associated with rate shifts.

Table 1.2: Associations between rate shifts and defensive trait states at nodes with rate shifts inferred by MEDUSA. The percentage of rate decreases with a trait present was compared to the percentage of rate increases with the trait present.

Trait	% state 1 at rate decrease	% state 1 at rate increase	Fisher's exact test (P-value)
Colony size	0%	33%	0.2605
Eye size	50%	92%	0.0833
Polymorphism	0%	8%	1
Spines	50%	42%	1
Sting	100%	67%	0.2451

1.4.6 Sister clade analysis

The sister clade analyses provided support for the impact of several traits on diversification. Among morphological defensive traits, colony size, eye size, and spines were associated with increased diversification rate ($P < 0.001$ for all traits; Fig.

1.3) and the sting was associated with decreased diversification rate ($P = 0.007$, Fig. 1.3), while polymorphism was not associated with diversification. Among ecological traits, broad foraging, and broad nesting were both associated with increased diversification rate ($P < 0.001$ and $P = 0.002$, respectively), while ground foraging and nesting and arboreal foraging and nesting were all associated with decreased diversification rate ($P < 0.001$, $P = 0.016$, $P = 0.035$, and $P < 0.001$, respectively; Supplementary Fig. A.11). Diet was not associated with diversification.

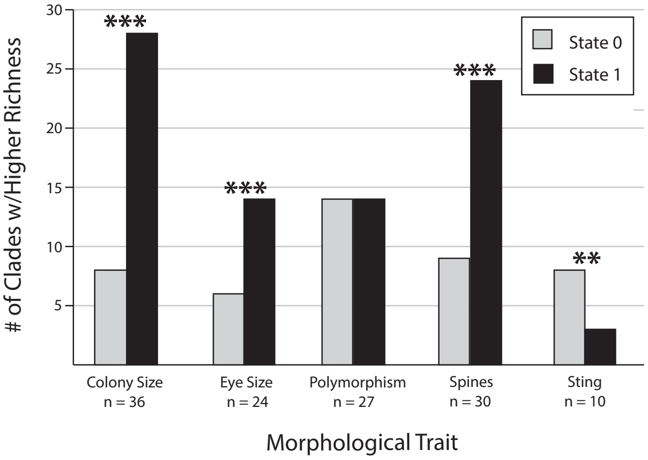

Figure 1.3: Results of sister clade analysis of morphological traits and diversification. An association between diversification and trait states (0 or 1) was assessed using a model-based likelihood ratio test (Paradis 2011; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

1.4.7 Trait correlation

Among the 55 phylogenetically informed pairwise tests for correlation, the model that included correlated transition rates was favored for 30 pairs of traits (Supplementary Table S1.9). These included seven morphological trait pairs and 23 morphological-ecological trait pairs. Among these 30 correlated trait pairs, the parameter restriction test found that the model with a difference in a tested transition rate was significantly favored for 18 trait pairs (Supplementary Table S1.9, Fig. 1.4). Among morphological traits pairs, the transition rates in the favored models supported a positive association between large eye size and large colony size as well as between large eye size and spines. The transition rates in the favored models also supported negative associations between the sting and all four of the other traits (large colony size, large eye size, polymorphism, and spines).

Among the 23 correlated morphological-ecological trait pairs, the parameter restriction test found that the model with a difference in a tested transition rate was significantly favored for 12 pairs (Supplementary Table S1.9). Eye size was positively associated with omnivory, and negatively associated with a predacious diet and ground nesting. Polymorphism was both positively and negatively associated with an herbivorous diet, and negatively associated with a predacious diet. Spines were positively associated with an herbivorous diet, and both positively and negatively associated with broad foraging. A sting was positively associated with a predacious diet but negatively associated with arboreal foraging and an herbivorous diet. Colony size was positively associated with an herbivorous diet, and negatively associated with a predacious diet.

1.5 Discussion

Species diversification and morphological evolution are shaped by diverse processes, including niche adaptation (Evans et al., 2009; Ricklefs, 2010), developmental constraints (Porto et al., 2015), key innovations (Rainford et al., 2014; Weber & Agrawal, 2014), interspecific competition (Rabosky, 2013; Price et al., 2014), and predation (Langerhans et al., 2004; Arbuckle & Speed, 2015). Here, we assessed the impact of evolutionary trade-offs among defensive traits on the evolution of the ants, an ecologically dominant clade of insects exhibiting remarkable morphological

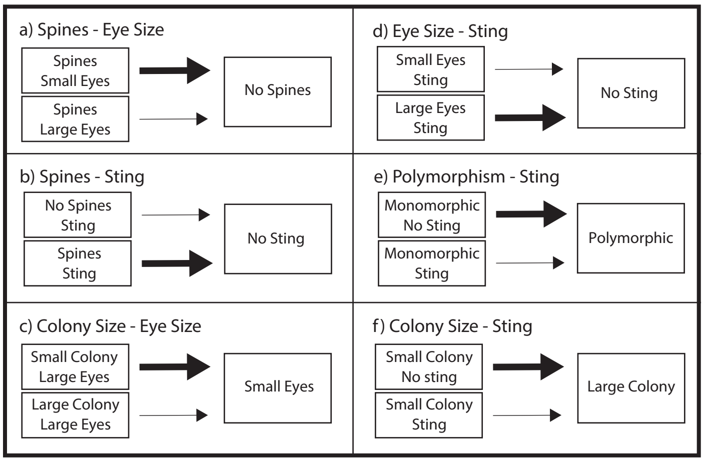

Figure 1.4: Results of the parameter restriction tests among significantly correlated morphological trait pairs. The 8-parameter model was compared to 7-parameter models in which one pair of parameters are constrained to be equal (see Methods). In (A)–(F), among all parameter pairs investigated, the unconstrained parameter difference that significantly increased model likelihood is shown. A thicker arrow indicates the transition parameter that is larger than its corresponding paired parameter in the model favored by the test.

diversity (Hölldobler & Wilson, 1990). We inferred the most complete phylogeny of ants to date, covering 82% of all currently recognized ant genera, and produced the first family-wide database of morphological and ecological traits that includes every described genus with a known worker caste (Supplementary Table S1.4). Using this phylogeny and database of traits, we implemented a number of analyses to determine patterns of diversification across ants, assess the evolutionary lability of multiple ecological and morphological traits as well as their association with diversification, and detect correlations between these traits to test the hypothesis that the evolution of defensive traits is constrained by a trade-off.

1.5.1 Trait evolution in ants

Three of our five morphological defensive traits exhibited significant directionality in losses and gains. In particular, eye size increases were 50% more common than decreases, spine loss was more than two times as common as spine gain, and the loss of a sting occurred repeatedly while no gains of a sting were inferred (Supplementary Table S1.7). Furthermore, evolutionary lability varied considerably—eye size, polymorphism, and spines all changed more than four times as often as the sting, which was highly stable. The most labile trait is colony size, with 238 inferred changes across the 271 genera (Supplementary Table S1.7). However, despite the large number of changes in colony size across the tree, large colony size in the subfamilies Dorylinae, Dolichoderinae, Formicinae, and Pseudomyrmecinae arose only once in each subfamily with subsequent losses in each clade, based on node reconstructions (Supplementary Fig. A.3). This pattern of variation in colony size evolution is particularly interesting, as colony size has been shown to be the ecological equivalent of body size in ants (Kaspari & Vargo, 1995; Hou et al., 2010; Dornhaus et al., 2012). In fact, a recent study found that macroevolutionary trends in colony size partially follow the predictions of a hypothesis initially developed to explain trends in body size evolution, where large colony sizes are generally maintained and size changes occur over relatively incremental steps rather than larger jumps (Burchill & Moreau, 2016). Body size is linked to diversification rate in many taxa (Maurer et al., 1992; Wollenberg et al., 2011; Rabosky et al., 2013), and is a key feature of several ecological and evolutionary theories such as Bergmann's rule (Meiri & Dayan, 2003), which has been observed for colony size in ants (Kaspari & Vargo, 1995). Given the general pattern of repeated evolution of large colony size across our phylogeny as well as recent evidence of the importance of colony size trends in ant evolution, investigations of colony size evolution on a broad scale across ants, including comparisons between colony size versus body size, is likely to be a promising avenue for future research.

We also detected differences in evolutionary lability among the three ecological traits of diet (herbivorous, predacious, or omnivorous), nesting (arboreal and/or ground), and foraging (arboreal and/or ground). Diet appears to be more stable than spatial ecological niche, with a trend toward specialization (predation or herbivory),whereas spatial ecological niche (nesting and foraging locations) changes much more often, but with no significant trends in directionality. The trend toward diet specialization is consistent with a previous study on diet transitions in phytophagous insects, which found that the transition rate from diet generalization to specialization was greater than the reverse transition rate (Nosil, 2002). Furthermore, our finding of frequent evolutionary changes in nesting location is consistent with a recent study of habitat changes across all ants (Lucky et al., 2013). It is possible that specializing in diet minimizes interspecific competition, while frequent changes in spatial niche, including many niche expansions, decrease the likelihood of extinction due to stochastic environmental change (Futuyma & Moreno, 1988; Jablonski, 2008).

1.5.2 Defensive traits and diversification

We find support for an association between some defensive traits and shifts in rates of diversification. In particular, large eye size and large colony size were associated with rate increases while the presence of a sting was associated with rate decreases. Although the statistical support for these trends was modest to weak, our sister clade analysis found a significant association between both large eye size and large colony size and elevated rates of diversification as well as a significant association between a sting and decreased rates of diversification (Fig. 1.3), consistent with the trends from the rate shift analysis. The sister clade analysis also found elevated diversification significantly associated with spinescence, but did not find a significant association between polymorphism and diversification (Fig. 1.3).

There are several mechanisms that could cause spines, large eye size, and large colony sizes to increase diversification rate. One explanation for why certain defensive traits might drive diversification is the escape-and-radiate hypothesis, which states that species that evolve traits allowing them to escape from natural predators may be more able to evolve into novel niches and subsequently radiate (Schluter, 2000; Arbuckle & Speed, 2015). The predictions of this hypothesis suggest that both spines and the increased visual acuity afforded by large eye size allow species to avoid predation, while large colony size diminishes the cost of individuals lost to predation, driving increased diversification. In fact, it is notable that the two ecological traits associated with elevated diversification represent ecological niche expansion, namely broad foraging and broad nesting, while some niche specialization (ground foraging and nesting) is associated with lower rates of diversification (Supplementary Fig. A.11). This expansion of ecological niche can promote diversification through ecological opportunity (Moreau et al., 2006; Yoder et al., 2010; Price et al., 2014) consistent with predictions of the escape-and-radiate hypothesis. Our results are also consistent with recent evidence that herbivory is not associated with elevated diversification in Hymenoptera despite an association in many other insect orders (Wiens et al., 2015).We suggest that diversification in ants is promoted by morphological traits that buffer species against enemy attack and an expansion in ecological niche traits that may promote speciation and/or slow extinction. However, further work on ecologically relevant morphological traits is needed to elucidate the mechanistic forces driving trait-based diversification in ants.

1.5.3 Evolutionary trade-offs in defensive traits

The results of our corHMM analyses support the existence of a trade-off between investing in a sting versus a suite of traits including polymorphism, large eye size, large colony size, and spines (Fig. 1.4). In particular, the loss of a sting is more likely when spines or large eyes are present, and the gain of polymorphism or large colony size is more likely when a sting is absent. Furthermore, the loss of spines is more likely when eyes are small, and the loss of large eyes is more common when colony size is small. These data therefore support an evolutionary trade-off between a sting and the other defensive traits tested, and also suggest that spines, large colony size, and large eye size may form a defensive suite.

This finding of a suite of traits that trade off with a sting is consistent with recent studies in plants that suggest that defense is characterized more by defense "syndromes" of correlated traits rather than trade-offs between individual traits (Agrawal, 2007; Agrawal, 2011). These traits that trade off with a sting may therefore be interdependent. For example, an increase in colony size may allow for the division of labor through polymorphism, which would otherwise be too costly for species with smaller colonies (Tschinkel, 1988; Thomas & Elgar, 2003). Similarly, large eyes may be more beneficial when colony sizes are large due to a heightened risk of exposure and subsequent predation. Furthermore, there may be a trade-off between a "strength-in-numbers" approach (e.g., large colony size) and individual worker trait investment (e.g., sting) for active defense strategies.

Considered in conjunction with our trait-based diversification results, the trade-offs that we find here have interesting macroevolutionary implications. Spines, large colony size, and large eyes are all associated with elevated rates of diversification and trade off with a sting, which is associated with decreased rates of diversification. Thus, selection to evolve a sting may be beneficial in the short term but ultimately suppress rates of diversification over evolutionary time, as having a sting constrains a given clade from evolving other traits that are drivers of diversification. This constraint may be driven by the costly production of a sting and its associated chemicals, as well as the energy cost resulting from actively stinging a given attacker, and these additional costs may not be fully compensated for by the alternative use of a sting in prey capture. Therefore, our data suggest that sting production at the expense of other defensive traits may be a type of "evolutionary dead end" in ants (Agnarsson et al., 2006), and that dynamics at the species or population level (e.g., selection for a sting) may generate unexpected evolutionary dynamics at broader taxonomic levels (e.g., lower rates of diversification in one clade relative to other clades) (Jablonski, 2008).

1.6 Chapter Conclusion

We have presented here the first multitrait investigation of morphological evolution across all ants, using a well-sampled molecular phylogeny to address the role of trait trade-offs in ant evolution. Our trait database is the first to include information on every described extant genus with a known worker caste, and we hope that making the database public and editable will serve to enhance further research. We find strong evidence for an evolutionary trade-off between sting production and a suite of defensive traits including spines, large colony size, and large eyes, supported by both phylogenetic and nonphylogenetic analyses. Our results suggest that defensive traits significantly influence macroevolutionary patterns in ants. Given that there is little data on the particular ecological roles of these traits for the majority of ants (Lach et al., 2010), we emphasize the need for more work on the functional ecology of defensive traits in this highly diverse and ecologically important group of insects. Such work is crucial to expand our understanding of ant evolution, from ecological process to evolutionary outcome.

2 Spine and dine: A key defensive trait promotes ecological success in spiny ants

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2.1 Abstract

A key focus of ecologists is explaining the origin and maintenance of morphological diversity and its association with ecological success. We investigate potential benefits and costs of a common and varied morphological trait, cuticular spines, for foraging behavior, interspecific competition, and predator-prey interactions in naturally co-occurring spiny ants (Hymenoptera: Formicidae: *Polyrhachis*) in an experimental setting. We expect that a defensive trait like spines might be associated with more conspicuous foraging, a greater number of workers sent out to forage, and potentially increased competitive ability. Alternatively, consistent with the ecological trade-off

hypothesis, we expect that investment in spines for anti-predator defense might be negatively correlated with these other ecological traits. We find little evidence for any costs to ecological traits, instead finding that species with longer spines either outperform or do not differ from species with shorter spines for all tested metrics, including resource discovery rate and foraging effort as well as competitive ability and anti-predator defense. Spines appear to confer broad anti-predator benefits and serve as a form of defense with undetectable costs to key ecological abilities like resource foraging and competitive ability, providing an explanation for both the ecological success of the study genus and the large number of evolutionary origins of this trait across all ants. This study also provides a rare quantitative empirical test of ecological effects related to a morphological trait in ants.

2.2 Introduction

Providing explanations for the origin and maintenance of morphological diversity and identifying subsequent consequences for ecological success are longstanding goals of ecology. The evolution of particular morphological traits can determine outcomes of major ecological processes as wide-ranging as establishment into novel niches (Azzurro et al., 2014), intra- and interspecific competition (Bennett et al., 2016), predator-prey interactions (Green & Côté, 2014), and mate choice (Roeder et al., 2019). This multitude of potential axes of selection within an environment that might influence morphological trait evolution and expression suggests that any given trait adapted for one ecological function may carry costs for, and trade off with, other functions (Stearns, 1989; Kneitel & Chase, 2004). Understanding the ecological benefits and costs of morphological traits requires quantitative assessments of trait function, and such assessments have been especially productive in plant systems (Tilman & Pacala, 1993; Mole, 1994; Agrawal & Fishbein, 2006), where experiments are generally easier than in animal systems. The depth of studies on functional traits in plants has allowed more complex theorizing about interacting suites of traits as opposed to, for example, univariate trade-offs (Koricheva et al., 2004; Agrawal & Fishbein 2006; Agrawal 2007), and highlights the efficacy of pursuing quantitative functional trait experimental methods to understand trait-fitness relationships in diverse clades of interest.

Here, we investigate the role of a common morphological trait, cuticular spines ("spinescence"), on foraging behavior, interspecific competition, and predator-prey dynamics in *Polyrhachis* ants in a lab setting. Spines vary remarkably across ant species, sometimes exhibiting an extreme range of phenotypes within a single genus, from no spines to multiple large and curved thorn-like projections reaching lengths matching the length of the entire thorax (Fig. 2.1; Sarnat et al., 2016). Importantly, ant spines are typically greatly reduced or absent in the reproductive caste (queens and males), and thus their function likely differs from well-studied cases of spines undergoing sexual selection (e.g. Emlen, 1997). Recent work shows that spinescence is associated with elevated species diversification rates across all ants (Blanchard & Moreau, 2017), and spines appear to confer some defense against vertebrate predators (Ito et al., 2016). Pekár et al. (2017) find decreases in predation from both vertebrate and invertebrate predators in a study that bins all defensive traits – including spines – into a single trait, but Mikolajewski et al. (2006) demonstrate that spines in dragonfly larva are ineffective in defending against invertebrate predators. These results suggest that a greater ecological understanding of spine function and impact on fitness would significantly contribute to our understanding of drivers of ecological success, morphological divergence, and species diversity in ants, a globally dominant group of insects.

While experimental tests of morphological trait function are rare in ant research

Figure 2.1: Morphological variation in the ant genus *Polyrhachis*. Species (and photo credit), from top left: *P. boltoni* (Michael Esposito), *P. robsoni* (Will Ericson), *P. deceptor* (April Noble), *P. loweryi* (Will Ericson), *P. ornata* (Michele Esposito), *P. lata* (Cerise Chen), *P. hippomanes* (April Noble), *P. armata* (Estella Ortega), *P. ypsilon* (Estella Ortega). From www.antweb.org under a Creative Commons Attribution License. Accessed 13 June 2019.

(e.g. Poulsen et al., 2002; Larabee & Suarez, 2015; Ito et al. 2016), existing work suggests some specific associations between traits and ecological functions, for example between head, eye, limb, and total body size and diet, foraging strata, and guild (Weiser & Kaspari, 2006; Silva & Brandão, 2010; Gibb et al., 2015). Utilizing lab experiments, Larabee and Suarez (2015) found fitness increases from high-powered mandibles that allow trap-jaw ant workers to propel themselves away from antlion attacks. Field and lab work on tropical canopy ants suggests species assembly is mediated, at least in part, by body size variation (Fayle et al., 2015), while Retana et al. (2015) showed that several morphological traits (e.g. worker polymorphism) were associated with foraging strategy, behavioral dominance, and other ecological traits. Given such known associations between morphological and ecological traits in ants, it is likely that many traits carry benefits and costs within and between ant species.

We focus on competition and predation as two selection pressures likely to drive adaptation in ants. Numerous studies have used field and lab methods to better understand ant competition. One common approach is to address a putative tradeoff between resource discovery and resource dominance as different strategies used to minimize competitive interactions between ant species (Davidson, 1998; LeBrun & Feener, 2007; Parr & Gibb, 2012; Bertelsmeier et al., 2015). Field studies have targeted the role of dominance hierarchies in structuring communities (Savolainen & Vepsalainen, 1988; Stuble et al., 2013), as well as the mechanisms involved in competitive exclusion by native ants (McGlynn & Parra, 2016) and invasive ants (Holway, 1999). Lab studies have provided support for theorized species assembly rules in tropical canopy ants (Fayle et al., 2015) and the influence of intraspecific variation in competitive dynamics (Thomas et al., 2004; Lichtenstein et al., 2015).

Relative to competition, the influence of predation on ant ecology and evolution has been largely underexplored (Cerdá et al., 2013). The studies that do exist suggest predator-prey dynamics are a promising area of research, providing explanations for important behaviors like nest relocation (McGlynn et al., 2004) and significant variation in morphological traits like mandibular shape (Larabee & Suarez, 2015) and cuticular spines (Ito et al., 2016). Understanding compelling putative top-down mechanisms of diversification, like that proposed by the escape-and-radiate hypothesis (Ehrlich & Raven, 1964), require experimental work. Therefore, extant studies in ants and other systems should motivate a greater focus on the effects of predators on ecological interactions and species diversification.

In this study, we test for benefits and costs of spines through a novel investigation that directly connects a morphological trait to multiple ecological trait outcomes in this insect group. Under an expectation that spines are adapted to repel predators (Dornhaus & Powell, 2010), we predict that spines are positively associated with anti-predator defense. Furthermore, we predict that the associated release from predator pressure allows workers with spines to exhibit increased, conspicuous resource foraging abilities and potentially enhanced abilities in other ecological traits like competitive ability as well. This second prediction can be explained through two plausible mechanisms. First, if predation exerts significant selection for predator avoidance behaviors that decrease foraging efficiency by promoting inconspicuous foraging strategies such as foraging in smaller numbers or using circuitous foraging routes, then we predict that selection for anti-predator defensive spines in a given population will promote conspicuous foraging traits like persistent occupancy of a foraging area and a higher number of foraging workers. Second, if such a population no longer experiences significant energetic loss from predation, a colony may be able to invest that energy into other key traits like competitive ability (e.g. aggression) and thus invest more in such traits relative to competitors that do not benefit from spine defenses.

However, despite the expected mechanisms described above, various potential costs of spine production may alternatively drive a negative association between anti-predator defense and other ecological traits including resource foraging and competitive abilities. The energy expended to produce spines, which in addition to cuticle can also contain muscle tissue (Sarnat et al., 2017), may trade off with energetic investment elsewhere (e.g. number of workers produced or muscle production in the legs). Particularly in the more extreme trait states, spines may reduce maneuverability for workers in their environment and in competitive interactions. Wilson (1959) posited that spines reduce the number of nesting spaces available to a species, as the more confined spaces in twigs and subterranean habitats may preclude occupancy by workers bearing such rigid, protruding structures. This constraint on movement might explain a negative association between spines and foraging abilities (such as resource discovery rate) or ability in competitive interactions. Furthermore, we might expect that ant species investing in defense invest less in competitive ability, a trade-off found in many other taxa (cotton plants: Karban et al., 1989; flies: Kraaijeveld & Godfray, 1997; crustaceans: Wellborn, 2002; algae: Yoshida et al., 2004; mosquitofish: Langerhans, 2009; salamanders: Urban & Richardson, 2015). Notably, such costs may exert selection against spines in a population, or a clade of species, on a different timescale than benefits exerting selection for spines, and temporal differences in selection may explain the repeated gains and losses of spines across the ant tree of life (Blanchard & Moreau, 2017).

Thus, to evaluate the potential ecological benefits and costs of spines, we ask: (1) Do species with spines invest more (or less) time and effort foraging for resources? (2) Is there a positive (or negative) relationship between spines and competitive ability? (3) Do species investing in spinescence have higher (or lower) survival under predator-prey conditions? Our work provides a quantitative empirical test of performance related to a morphological trait in ants as well as an assessment of morphological trait-mediated ecological trade-offs and potential trait-based drivers of ecological success in a diverse insect group.

2.3 Methods

We worked at the Xishuangbanna Tropical Botanical Garden (XTBG) in southern Yunnan province in southern China. XTBG and the surrounding regions in Xishuangbanna experience a tropical climate and are part of the Indo-Burma biodiversity hotspot (Myers et al., 2000). Correspondingly, Yunnan is home to the richest ant fauna in China with over 450 documented species (AntMaps.org; accessed February 2019; Janicki et al., 2016). One of the most speciose and morphologically diverse ant genera in this region is the spiny ant genus *Polyrhachis* Fr. Smith, with around 30 species currently known from the province (AntMaps.org). Most *Polyrhachis* species contain some number of exoskeletal spines up to one pair each at three locations along the mesosoma with an additional pair sometimes located on the petiole, although some species have no spines (Fig. 2.1). All species exhibit worker monomorphism. XTBG is well-suited for this work as numerous *Polyrhachis* species exhibiting a range of spinescence overlap in microhabitat at this location (Guénard & Dunn, 2012), and several species often nest in close proximity and share similar nesting behavior, using larval silk to construct nest structures between or on the underside of leaves (Robson & Kohout, 2007). Furthermore, most if not all *Polyrhachis* species have a diet low on the trophic scale that relies on Hemipteran mutualists, plant exudes, and other opportunistically acquired sugary resources in the environment (Liefke et al., 1998; Staab et al., 2017). This overlap in geographic location, microhabitat, and niche makes it likely that these species regularly interact in their environment, experiencing shared selection pressures from interspecific competition and predation.

During June and July of 2017 and 2018, we collected *Polyrhachis* colonies in and around XTBG. We kept the colonies in the field station lab in plastic containers (17 x 11.5 x 10 cm), and regularly provided each colony a standard liquid sugar diet (50% sugar/50% water solution) as a rough approximation of their natural sugary food sources. Species were identified in the lab using the primary literature (Xu, 2002a; Xu, 2002b; Kohout, 2010; Kohout, 2014) and images from AntWeb.org (accessed June-July 2017). Species voucher specimens were deposited in the Field Museum of Natural History in Chicago, IL, USA, and the Southwest Forestry University Specimen Hall in Kunming, Yunnan, China.

2.3.1 Resource discovery rate, foraging effort, and competitive ability trials

We first conducted resource discovery trials with 89 colonies, representing 11 *Polyrhachis* species (Supplementary Table S2.1). We explored the effect of spinescence on time and worker investment by testing associations between spines and resource discovery rate and foraging effort. Approximately 48 hours before a trial for a given colony, we removed the food from the colony's container. At the start of the trial, we removed the lid from the colony's container and placed it into a larger bin filled with 2-3 cm of water to create a moat (preventing escape) and let it sit for 5 minutes for colony acclimation. We then set a smaller container, with food (50% sugar/50% water solution), into a second larger bin filled with water, and used standardized manufactured wood pieces (chopsticks) as a bridge to connect this food chamber to the colony container (Supplementary Fig. B.1). Following attachment, we recorded the number of ants present in the food container every minute for 60 minutes. We noted the time until the first ant entered the food chamber ("Discovery Rate"), and two metrics of foraging effort: colony presence/absence in each one-minute time bin with "present" indicating at least one ant present in the food chamber ("Worker Presence"), and number of workers present in the food chamber during each one-minute time bin ("Worker Number"). We also documented species identity, colony size (counted manually following all trials), body size (determined for a species based on the diagonal length of the mesosoma, Weber's length, for one representative worker), and spine length (determined for a species based on the combined length of spines across one half of the mesosoma and petiole for one representative worker, Supplementary Table S2.1). While our metric for spine length is a combined variable that does not necessarily capture the full variation of spine morphologies in the genus, we believe it is a good first approximation of overall spinescence.

We then conducted multi-species interspecific competition trials, which served as a broad test of spine-mediated competitive ability in *Polyrhachis.* These included 72 colonies from 11 different species (Supplementary Table S2.1), with pairings between colonies randomized except for an attempt to maintain similar estimated colony sizes and collection date between species pairs. Our experimental design and trial methods matched the resource discovery trials, except paired colonies were attached to the same food container. We documented the number of ants present in the central chamber and in the opposing colony's container every minute for 60 minutes, for both colonies in a given trial. We summed the proportion of the colony present in the central chamber and in the opposing colony's container, averaged over the trial period and across trials within a species, and used this value to estimate competitive ability (see "Statistical tests", below).

Although a multi-species approach is ideal for identifying effects from spines distinct from other species effects, our multi-species trials suffer from low sample sizes for several species and thus limited statistical robustness overall. Therefore, we also utilized a two-species approach. From the multi-species trials, we identified two focal species: *Polyrhachis flavicornis*, with two short petiolar spines, and *P. laevigata*, with four medium-length spines on the propodeum and petiole (Fig. 2.2a). These species are naturally co-occurring and have very similar body size, overall appearance, arboreal nest architecture, and geographic ranges (Fig. 2.2b; AntMaps.org). Colonies of both species are monogynous (single queen) and monodomous (single nest dome), unlike at least two species in the multi-species trials, minimizing potential associated confounding factors. Furthermore, we could collect a relatively large number of individual colonies of each species, as they are both common in our collection area. Therefore, *P. flavicornis* and *P. laevigata* were well-suited for testing our hypotheses while minimizing the likelihood of various other confounding factors, although we acknowledge that two-species tests remain limited in their utility to robustly assess adaptive hypotheses (Garland & Adolph, 1994).

The two focal species, *P. flavicornis* and *P. laevigata*, were used for additional trials using the same experimental design and methods as for the multi-species discovery trials. The two-species trials included 30 colonies from each species, for a total of 60 colonies (Supplementary Table S2.2). As with the multi-species trials, "Discovery Rate", "Worker Presence", and "Worker Number" data were collected over 60-minute trials. Furthermore, to test the impact of spines on interspecific competition and the potential for an ecological trade-off between anti-predator defense and competitive ability, we conducted competition trials as we did for the multi-species dataset, pairing colonies to minimize differences in estimated colony size and collection date (n = 30 pairs). Through comparing the resource discovery rate and foraging effort results when colonies were paired versus when they were alone, we could assess shifts in abilities, where a significant increase or decrease in outcomes

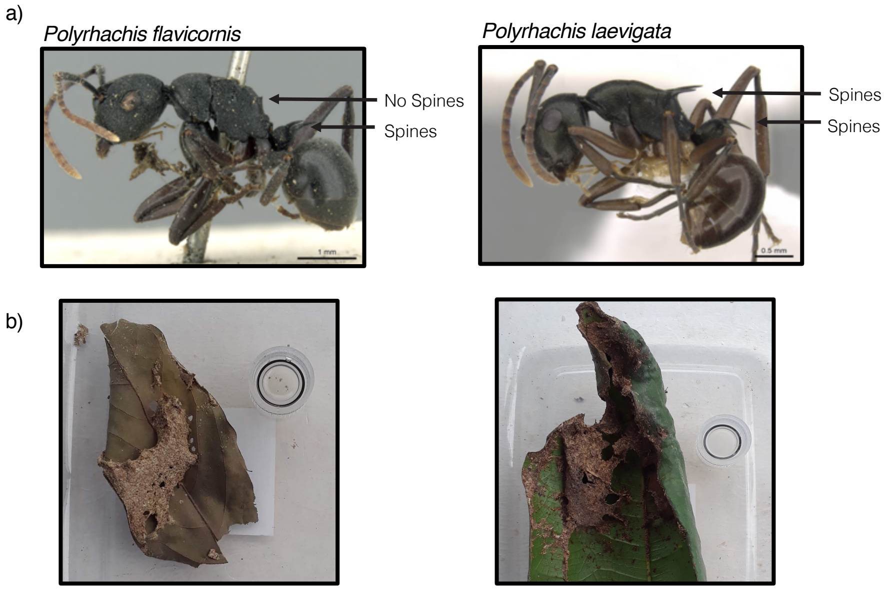


Figure 2.2: Comparison between *P. flavicornis* and *P. laevigata* (a) morphology, and (b) nests (after collection). Image credit: (a) Estella Ortega and Michele Esposito, from www.antweb.org under a Creative Commons Attribution License. Accessed 13 June 2019. (b) B.D. Blanchard.

for one species (but not the other) would signal superior or inferior competitive ability, respectively.

2.3.2 Anti-predator defense trials

To assess the function of spines in repelling invertebrate predators, in August 2018 we hand collected adult individuals of *Siler semiglaucus*, a common and widespread ant-specialist jumping spider that preys on adult ants (Jackson & Olphen, 1992; World Spider Catalog, accessed August 2018), at XTBG. Although Mikolajewski et al. (2006) found that spines were ineffective against invertebrate predators of aquatic dragonfly larvae, our terrestrial system is significantly different; furthermore, ant spines may present a physical barrier that limits the ability of jumping spiders to access the main body cavity at vulnerable joints along the body. We collected individuals of *S. semiglaucus* by beating shrubs over an upturned umbrella, and placed each spider in individual plastic tubes (10 x 4 cm) with a single flower petal to maintain humidity. After approximately three days (to ensure interest in prey), we placed a single spider into a small plastic box (10 x 10 x 6 cm) with an ant worker from one of the two focal species (*P. laevigata* and *P. flavicornis*; n = 11 and n = 12, respectively) and assessed ant survival after 1 and 24 hours. As controls, we placed 10 ants of each species in individual boxes (1 ant/box) without spiders present.

2.3.3 Statistical tests

For the multi-species resource discovery rate and foraging effort trials and the two-species resource discovery rate, foraging effort, and competition trials, we utilized generalized linear models (GLMs) and generalized linear mixed models (GLMMs) using the "glm" and "glmer" functions in R packages "stats" and "lme4" (Bates et al., 2015; R Core Team, 2018). For the multi-species trials, we modeled Discovery Rate, Worker Presence, and Worker Number using GLMMs. We assumed Discovery Rate followed a geometric distribution, which measures the number of intervals (time bins) before the first "success" (i.e. appearance of the first worker in the food chamber). We assumed Worker Number per time bin followed a binomial distribution, which measures the number of successes (workers in the food chamber) out of the total colony size. Similarly, we assumed the response for Worker Presence, a binary trait where "0" represents absence of any workers and "1" represents presence of at least one worker, followed a Bernoulli distribution. For all GLMMs, spine length and body size were modeled as fixed effects while species identity was treated as a random effect. For Discovery Rate and Worker Presence, colony size was also included as a fixed effect. For Worker Presence and Worker Number, time bin was added as a fixed effect, as we expect progression of time during the trial to be important and want to account for this effect. We also treat specific colony identity as an additional random effect.

We conducted similar analyses for our two-species resource discovery rate, foraging effort, and competitive ability trials. As we were interested in comparing *P. flavicornis* (short spines) and *P. laevigata* (medium spines), we modeled species as a fixed effect rather than random effect. As a result, the model for Discovery Rate was a GLM rather than a GLMM. Furthermore, to assess behavioral differences resulting from competition, results from discovery rate and foraging effort (alone) trials were compared to results from competitive ability (paired) trials for the same species for each of the two species, using the same GLMM/GLM framework for Discovery Rate, Worker Presence, and Worker Number.

For all GLM/GLMM analyses, we confirmed, using Pearson's correlation coefficient in the base R function "cor", that all variables included in each model exhibited a correlation coefficient < 0.8 (Supplementary Table S2.3).

For the multi-species test of competition, we used the Colley matrix (Colley, 2002). The Colley matrix, which was originally designed for ranking football teams but does not carry any sports-specific assumptions, is well-suited to situations with a large number of competitors but a very small number of pairings out of the total possible number of pairwise competitive events (LeBrun & Feener, 2007; Stuble et al., 2013). This method uses "win-loss" data and incorporates the relative strength of each competitor in ranking all competitors according to interaction outcomes. We considered each species as a competitor, and a "winner" for any given pairing to be the colony that had a higher percentage of the colony present in the containers averaged over the trial period. We then conducted a phylogenetic linear regression between the resulting Colley matrix metric values and relative spine length (spine length divided by body size), using the "phylolm" function in the R package "phylolm" (Ho & Ané, 2014). We utilize a dated molecular phylogeny of the genus, inferred using a genome-wide sequencing approach, that includes all taxa in this study (Blanchard & Moreau *in prep*).

To evaluate differences in ant prey survival, we used a chi-square test to compare differences between species after 1 hour and after 24 hours, with each box treated as an independent observation.

2.4 Results

2.4.1 Resource discovery rate and foraging effort

For our trials across multiple species (n = 9; Supplementary Table S2.4), Discovery Rate was not significantly associated with spine length, although there was a trend suggesting a positive relationship (Z = 1.51, P = 0.13; Fig. 2.3a). Discovery Rate was not associated with body size but was positively associated with colony size (Z = 0.06, P = 0.95; and Z = 2.54, P = 0.01; respectively), suggesting faster discovery ratess for species with larger colony sizes. Worker Presence (i.e. colony presence/absence in each one-minute time bin with "present" indicating at least one ant present in the food chamber) was significantly positively associated with spine length and Worker Number was somewhat significantly positively associated with spine length (Z = 2.165, P = 0.03; and Z = 1.74, P = 0.08; respectively; Fig. 2.3a), while neither were associated with body size (Z = -0.38, P = 0.70; and Z = -0.26, P = 0.80; respectively). Colony size was not associated with Worker Presence (Z = 1.58, P = 0.12), and both Worker Presence and Worker Number were positively associated with time during the trial (Z = 8.19, $P < 0.01$; and Z = 24.59, $P < 0.01$; respectively).

We observed that the two species in the *P. (Cyrtomyrma)* subgenus, representing

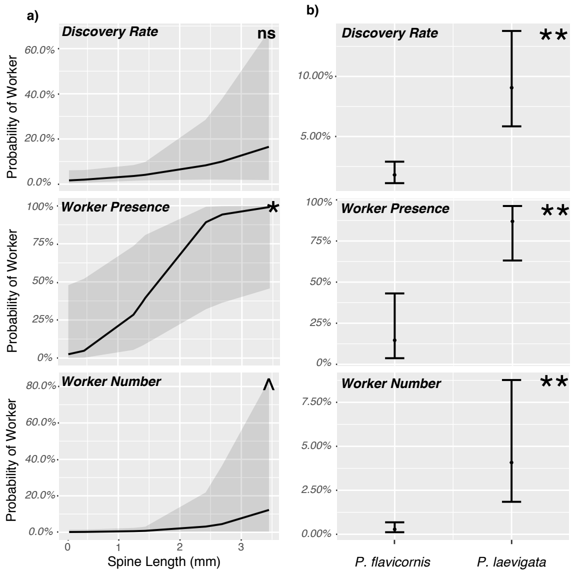


Figure 2.3: Resource discovery rate and foraging effort for (a) multi-species (n = 9, *Cyrtomyrma* excluded) trials and (b) two-species trials. Grey zones and bar lines indicate 95% prediction interval boundaries, i.e. the interval expected to contain 95% of future observations for a given spine length or species, respectively. ^ = $P < 0.1$, * = $P < 0.05$, ** = $P < 0.001$, ns = not significant ($P > 0.1$).

eight (9%) of the 89 colonies in our dataset, fell far outside the general trends across all 11 species. Furthermore, these species exhibit a distinct, extreme defensive behavior of rapidly dropping off leaves and onto the ground when even moderately disturbed by a vertebrate (pers. obs.). Given these observations, and our focus on morphological defenses as opposed to any alternative defenses, we excluded these eight colonies from our main results (reported above) but include them in Supplementary Fig. B.2 and Supplementary Table S2.4. Notably, the overall trends were similar, although less statistically supported, even when including the eight colonies from these two species exhibiting distinct, non-morphological defensive behaviors.

In the two-species trials (Supplementary Table S2.4), *P. laevigata*, the species with longer spines, had a significantly faster Discovery Rate and higher degree of Worker Presence and Worker Number compared to *P. flavicornis* ($Z > 3.48$, $P < 0.01$ in all cases; Fig. 2.3b). Colony size did not impact Discovery Rate ($Z = 0.20$,

$P = 0.84$) but was positively associated with Worker Presence ($Z = 2.40$, $P = 0.02$) in the two-species case. Time was positively associated with both Worker Presence and Worker Number ($Z > 15.73$, $P < 0.01$ for both).

2.4.2 Competitive ability

Our phylogenetic regression of Colley Matrix values across spine length for the multi-species trials did not support any significant association ($P = 0.89$, Fig. 2.4, Supplementary Table S2.5). In our two-species trials (Supplementary Table S2.6), our results differed for each species. For *P. flavicornis*, the species with smaller spines, we found no significant difference between alone and paired trials for Discovery Rate ($Z = 0.27$, $P = 0.79$) or Worker Presence ($Z =$ -1.34, $P = 0.18$) but did find higher Worker Number in the paired trials ($Z =$ -3.84, $P < 0.01$) (Fig. 2.5a). For *P. laevigata*, the species with longer spines, we found significant differences across all trial comparisons, with faster Discovery Rate ($Z =$ -3.05, $P < 0.01$) and higher Worker Presence ($Z =$ -7.58, $P < 0.01$) and Worker Number ($Z =$ -2.97, $P < 0.01$) in paired versus alone trials (Fig. 2.5b).

2.4.3 Anti-predator defense

We found that survival from predation by the spider *S. semiglaucus* tended to be higher for *P. laevigata*, the species with longer spines, than for *P. flavicornis*. This relationship was not statistically significant after one hour ($\chi^2 2 = 2.56$, $P = 0.11$), but was marginally significant after 24 hours ($\chi^2 = 3.16$, $P = 0.08$) (Fig. 2.6).

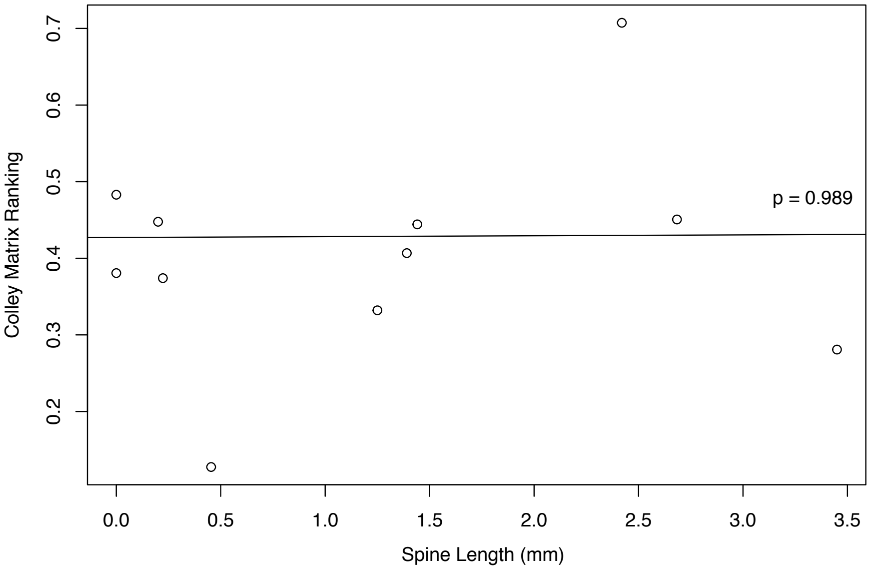


Figure 2.4: Competitive ability results from multi-species trials (n = 11) using a phylogenetic regression along Colley Matrix scores.

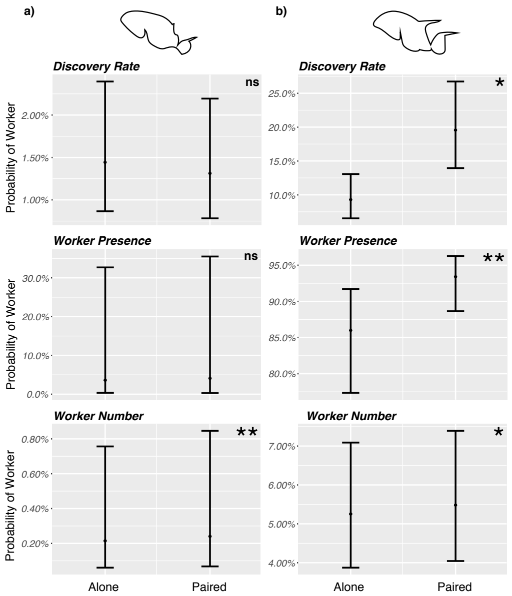


Figure 2.5: Resource discovery rate and foraging effort ("Alone") outcomes compared to competitive ability outcomes ("Paired") for (a) *P. flavicornis* and (b) *P. laevigata*. Bar lines indicate boundaries of the 95% prediction interval, i.e. the interval expected to contain 95% of future observations for a given species. * = $P < 0.01$, ** = $P < 0.001$, ns = not significant ($P > 0.1$).

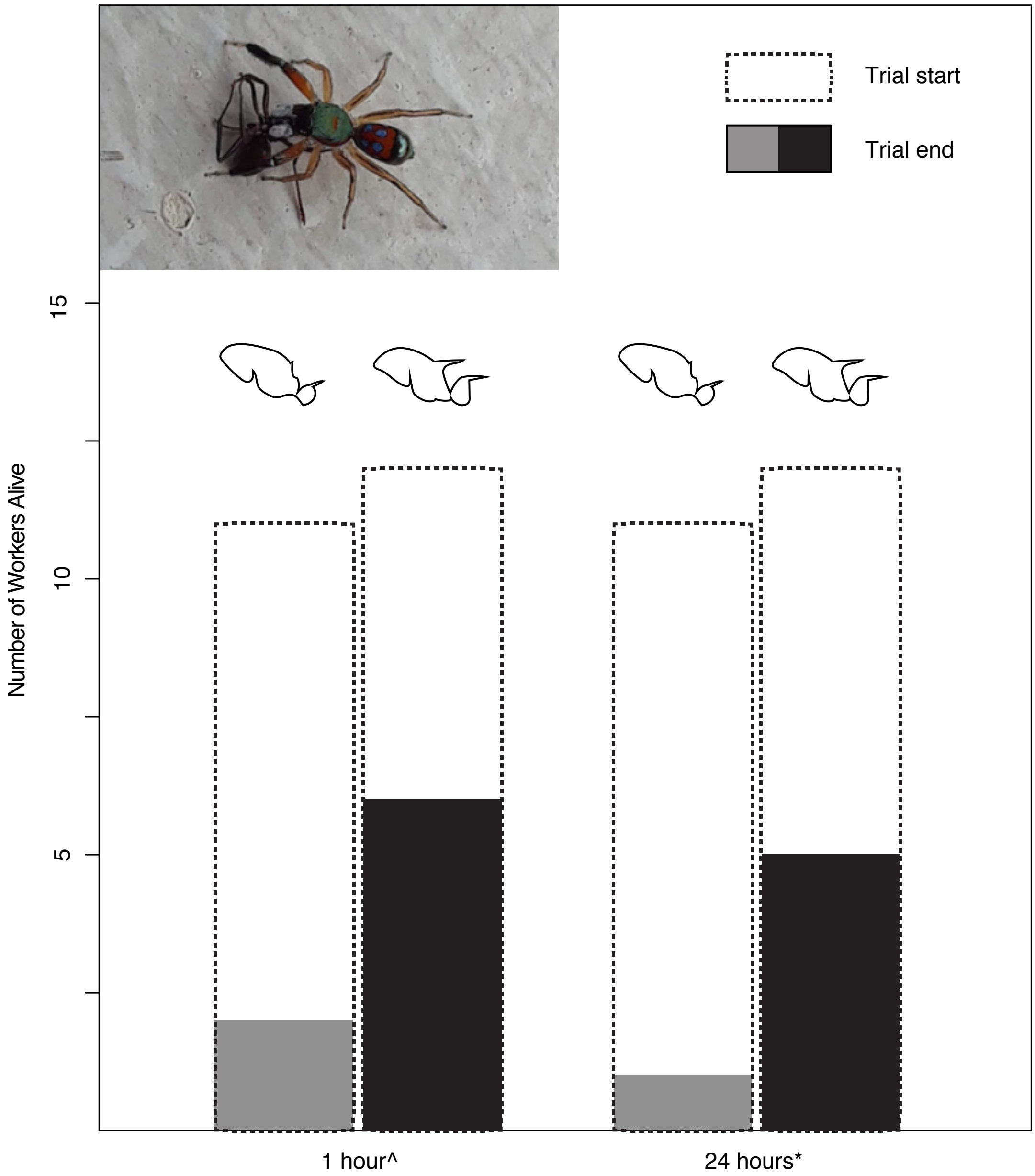


Figure 2.6: Differences in predator-prey survival for *P. flavicornis* and *P. laevigata* workers presented to the jumping spider *S. semiglaucus*. χ^2: $^\wedge P = 0.110$, $*P = 0.076$.

2.5 Discussion

We tested potential ecological benefits and costs of cuticular spines, a widespread and variable defensive morphological trait in ants. We found general support for a neutral to positive relationship between spines and all ecological traits we tested, including resource discovery rate, foraging effort, competitive ability, and anti-predator defense, with no costs detected. Our results thus support spines as a key defensive trait promoting broad ecological success, and do not support expectations of the ecological trade-off hypothesis. To our knowledge, this positive association between spines and resource acquisition traits and (potentially) competitive ability, not just anti-predator defense, has not been previously proposed in the literature and represents an intriguing area of future research.

Our finding of broader ecological trait benefits of an adaptive trait beyond the most proximate adaptive function highlights the wide-ranging potential impacts of morphological trait evolution. In fact, while the support for a positive association between spines and defense against our invertebrate jumping spider predator was rather modest (Fig. 2.6), the strongest result was a positive association between spines and foraging ability (Fig. 2.3). This dynamic is similar to other systems where release or escape from one selection pressure allows a species to invest in traits adapted to other selection pressures (Kitajima, 1994; Kinnison et al., 2003; Fulton et al., 2016). Our study suggests a potential mechanistic sequence that results in spines driving broad ecological success: Spines first confer anti-predator defense, which then reduces the need to hide from predators, subsequently allowing for more conspicuous foraging, which facilitates faster resource discovery and enhanced resource dominance, further conferring competitive advantages relative to less spinescent populations.

While we do not test trait-based diversification here, recent phylogenetic work on spines in ants suggests spines are an important morphological trait in ant evolution. One study proposed a link between extreme spinescence and occupancy of inland niches on islands in the hyperdiverse genus *Pheidole* (Sarnat & Moreau, 2011), while later work supported a link between spines and islands but not high-elevation habitats (Sarnat et al., 2017). Blanchard and Moreau (2017) demonstrated a positive association between spines and elevated diversification rates as well as high evolutionary lability for spines across the ant phylogeny. Considered together with these studies and previous work supporting ant spines as protection against vertebrate predators (Ito et al., 2016), our results suggest that spinescence serves as a generalized physical defensive trait with broad downstream ecological benefits potentially promoting high diversification rates in *Polyrhachis*, the fourth most diverse ant genus with 700 described species, and ants more broadly. However, more trait-based phylogenetic work is needed to detail the importance of spines for ant diversification, and in particular more robustly establish possible ecological mechanisms, like escape-and-radiate dynamics (Ehrlich & Raven, 1964; Arbuckle & Speed, 2015), linking defensive function and evolutionary radiation.

Although ecological trade-offs exist in many taxa and especially between competitive ability and anti-predator defense (Kneitel & Chase, 2004; Jessup & Bohannan, 2008), we did not find any spine-mediated trade-off between anti-predator defense and resource discovery rate, foraging effort, or competitive ability. The production of cuticular spines may be energetically cheap and/or developmentally simple, which may also explain the high evolutionary lability of the trait (Blanchard & Moreau, 2017), and future work could focus on a more mechanistic understanding of spine development and potential costs. In contrast to some plant species which exhibit phenotypic plasticity in increasing spine production when induced by herbivores (e.g. Young, 1987), spine production is canalized among the adult worker caste at a colonial level, possibly owing to consistency of predator pressure or low costs of trait production. Additionally, although competition and predation are fundamental ecological processes, other dynamics may exert greater constraining influences that would explain why, given their apparent broad benefits, spines have not evolved in all ant species and are, for example, largely unique to *Polyrhachis* among the Formicinae subfamily. Murrell and Juliano (2013) found that a competition-predation trade-off is not supported in some mosquitos, instead finding a colonization-competition trade-off. Supriya et al. (2018) report a positive, rather than negative, correlation between pre- and post-copulatory traits in warblers, possibly resulting from differential investment in overall fertilization success versus survival. Thus, a trade-off may exist between competition or predation and traits not included in our study, such as number of reproductives or colony longevity, etc. Still, given the prevalence of a competition-predation trade-off across many terrestrial and aquatic systems (Kneitel & Chase, 2004), our failure to detect evidence of such a trade-off is intriguing and warrants further study.

It is important to recognize that our experimental methods, in an effort to render the system tractable, also simplify the pressures that exist in a highly variable natural environment. Adding the structural complexity of the physical environment, including vertebrate predators, and allowing workers to compete in a less restricted spatial context may reveal dynamics that our methods could not detect. Furthermore, in “controlling for” colony size, we may also artificially constrain a species’ competitive ability that would normally compensate for smaller spines by having a larger colony size. Nevertheless, we view this study as a first step towards identifying mechanistic explanations for spines and ecological success in this group, and a good starting point for developing more complex theories and experimental methods.

Future research should target predator-driven dynamics potentially influencing morphological divergence as well as other ecological and evolutionary processes in ants. Such top-down influences on ant communities and species have been underexplored (Cerdá et al., 2013), and our study supports the importance of predator-prey dynamics for ants. Although difficult to execute, more sophisticated functional trait studies, like those seen in plants, will be necessary to test theories positing trait-based explanatory factors for community assembly as well as ecological and evolutionary success in ants and other diverse animal groups. Multi-species and multi-trait approaches, and in particular controlled trials with experimental manipulation of traits (Larabee & Suarez, 2015), are sure to enhance our understanding of the link between defensive traits, species interactions, and diversification in these groups. Most broadly, exploring the wider ecological implications of functional traits beyond their proximate adaptive function will promote a deeper understanding of the impacts of morphological traits in animal ecology.

3 Defensive spines are associated with large geographic range but not diversification in spiny ants (Hymenoptera: *Polyrhachis*)

3.1 Abstract

Several prominent evolutionary theories propose mechanisms whereby the evolution of a defensive trait or suite of traits causes significant shifts in species diversification rate and niche evolution. We investigate the role of cuticular spines, a highly variable morphological defensive trait in the hyperdiverse ant genus *Polyrhachis*, on species diversification and geographic range size. Informed by key innovation theory and the escape-and-radiate hypothesis, we predicted that species with longer spines would exhibit elevated rates of diversification and larger range sizes compared to species with shorter spines. To address these predictions, we estimated phylogenetic relationships with a phylogenomic approach utilizing ultra-conserved elements (UCEs) and compiled morphological and biogeographic trait databases. In contrast to the first prediction, we found no association between diversification rate and any

trait (spine length, body size, and range size). However, we recovered a positive correlation between spine length and geographic range size, suggesting that spines promote expanded geographic range. This study provides a rare investigation of the role of a defensive trait on geographic range size, and ultimately supports the hypothesis that defensive spines are a factor in increased range size in *Polyrhachis* ants. Furthermore, the lack of support for an association between spines and diversification, which contrasts with previous work demonstrating a positive association between spines and diversification rate, is intriguing and warrants further study.

3.2 Introduction

Defensive traits can play a key role in the evolution of a clade, promoting coevolutionary dynamics with predators (Edger et al., 2015; Brodersen et al., 2018), geographic range size contractions or expansions (Siemens et al., 2009; Luiz et al., 2013), niche specialization (Darst et al., 2004), and morphological divergence (Vamosi & Schluter, 2004; Kursar et al., 2009). Prominent evolutionary theories propose putative mechanisms whereby the evolution of a defensive trait or suite of traits results in significant shifts in species diversification rate and niche evolution. One such theory proposes that the evolution of a “key innovation” drives subsequent significant increases in diversification rates (Mayr, 1960), for example the association between the evolution of extrafloral nectaries, an ant-mediated defense, and elevated diversification in vascular plants (Weber & Agrawal, 2014). Similarly, the escape-and-radiate hypothesis proposes that escape from predators, deriving from the evolution of some defensive trait, drives cycles of diversification (Ehrlich & Raven, 1964). While the plant-butterfly herbivore system is the paradigmatic test case for this hypothesis (Suchan & Alvarez, 2015), Arbuckle & Speed (2015) find support for elevated speciation rates in frog clades that utilize aposematic coloration. Evaluating the influence of morphological defensive traits on macroevolutionary dynamics typically requires robust phylogenetic information and a clade that is diverse enough to include many phylogenetically-independent evolutionary shifts in the traits of interest (Maddison & FitzJohn, 2015).

The spiny ant genus *Polyrhachis*, a hyperdiverse clade of 700 described species (antcat.org; accessed February, 2020) with a broad geographic range spanning from Western Africa to Melanesia, exhibits remarkable variation in cuticular spine morphology. Spine trait states range from entirely absent to extreme lengths exceeding that of the thorax (Fig. 2.1), and recent work has established that such spines are likely adaptive defenses against vertebrate (and possibly invertebrate) predation (Ito et al., 2016; Pekár et al., 2017; Blanchard et al., *in press*). Furthermore, a broad investigation across all ants found that spines are highly evolutionary labile and associated with elevated diversification rates (Blanchard et al., 2017). *Polyrhachis* species also vary wildly in their geographic range even within the same subgenus, with some species restricted to one locality while others are distributed across much of the Oriental and Australasian regions (AntMaps.org; Guénard et al., 2017; Janicki et al., 2016). This insect genus is thus ideal for probing the relationship between a variable defensive trait and species diversification as well as geographic range size.

Ant phylogenetics has expanded into the field of phylogenomics through the use of RADseq (Fischer et al., 2015; Moreau & Wray, 2017), genotyping by sequencing (Winston et al., 2016), transcriptomics (Johnson et al., 2013) and, most recently, ultraconserved elements (“UCEs”; Faircloth et al., 2012; Branstetter et al., 2017a; Zhang et al., 2019). The use of UCEs has proven fruitful in ants and other animal groups, contributing to the phylogenetic resolution of the Hymenoptera (Branstetter et al., 2017b) and major ant lineages like Formicinae (Blaimer et al., 2015) and fungus-farming ants (Ješovnik et al., 2017), in addition to weevils (Van Dam et al., 2017), placental mammals (McCormack et al., 2012), ray-finned fishes (Faircloth et al., 2013), turtles (Crawford et al., 2015), and several other taxa (Zhang et al., 2019).

Here, we leverage the UCE sequencing approach to significantly expand the current phylogenetic coverage of *Polyrhachis* (Mezger & Moreau, 2016) and compile a dataset including spine length, body size, and range size information. With these data, we explore the impact of spine length evolution on species diversification and geographic range size while also accounting for body size, with a focus on expectations of defensive trait-based theories such as the escape-and-radiate hypothesis. In particular, we predicted that spines would be associated with both elevated diversification rates and expanded geographic range size, as expected if spines serve as defensive mechanisms that reduce extinction rates and promote spatial niche expansion due to a reduction of worker loss from predation.

3.3 Methods

3.3.1 Taxon sampling

Our initial data set for UCE sequencing included 181 samples from 180 *Polyrhachis* species including representatives from all 13 recognized subgenera and spanning the geographic range of the genus (Supplementary Table S3.1). Following sequence processing, cleaning, and filtering (see below), our final ingroup dataset included 161 tips from 160 species, including 12 of the 13 recognized subgenera (sequencing failed for the representative of the small *P. (Hirtomyrma)* subgenus; Supplementary Table S3.1). We obtained samples from specimens collected for a previous study (Mezger & Moreau, 2016), gifts and loans from collaborators, and the personal collections of authors of this study (B.D.B. and C.S.M.). See Supplementary Table

S3.2 for available collection information for each sample as well as voucher identity and depository location.

3.3.2 UCE sequencing workflow and bioinformatics

We conducted DNA extraction using the Qiagen DNEasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) following the protocol of Moreau (2014). After this step, library preparation, sample pooling, UCE enrichment, enrichment verification, final pooling, and Illumina sequencing followed the protocols described in Faircloth et al. (2015). All steps except for Illumina sequencing were conducted in the Field Museum Pritzker Laboratory, after which sample pools were sent to the University of Oregon GC3F iLab for Illumina sequencing on an Illumina HiSeq 4000 (150 bp paired-end reads; Illumina Inc., San Diego, CA, USA). The GC3F iLab also demultiplexed and converted the raw data into FASTQ format. For a few samples from a previous study (Mezger & Moreau, 2016), extracted DNA was already available, and thus we skipped the extraction step. We selected 15 taxa to use as outgroups from a previous phylogenomic study (Blaimer et al., 2015; Supplementary Table S3.1), downloaded raw FASTQ files through the Sequence Read Archive (Leinonen et al., 2011), and used these data in subsequent steps.

All processing steps, from cleaning to alignment, were conducted using PHYLUCE v1.6.6 (Faircloth, 2016) on the University of Chicago Research Computing Center Midway2 computing cluster. We cleaned and trimmed raw FASTQ reads using Illumiprocessor v2.0.2 (Faircloth, 2013), a wrapper program around trimmomatic (Bolger et al., 2014). We used Trinity v2.8.5 (Grabherr et al., 2011) to assemble the data, which due to version incompatibilities required using PHYLUCE v1.6.5 for this step only. We identified UCE loci by matching contigs to probes in the "hymenoptera-v2-ANT-SPECIFIC-uce" probe set (Branstetter et al., 2017) targeting 2,524 UCE loci that include both Hymenoptera- and ant-specific UCEs. After extracting UCE loci, we conducted alignment and further trimming used MAFFT v7.130b (Katoh et al. 2002) and Gblocks v0.91b (Castresana, 2000; Talavera & Castresana, 2007). Finally, we filtered the trimmed alignments based on taxon completeness (the percentage of taxa that must be included in a given alignment for that alignment to be retained). Preliminary investigations, including exploratory maximum likelihood inferences (see below), established that the 70% filtered set maximized completeness while retaining a relatively high total number of alignments. Through quality control steps in PHYLUCE, and initial maximum likelihood inferences, we determined that sequence quality for 20 samples was insufficient for inclusion. Thus, the final dataset processed through the PHYLUCE pipeline was our 176-taxon dataset (161 ingroup taxa and 15 outgroup taxa).

3.3.3 Phylogenomic analyses of 70% taxon complete matrix

Using the 70%-taxon-complete dataset, we inferred phylogenetic tree topologies through maximum likelihood (ML), Bayesian (BI), and gene-tree (GT) methods. For the ML analysis, we conducted inferences using RAxML-HPC BlackBox (Stamatakis, 2014) on the CIPRES computer cluster (Miller et al, 2010), designating outgroups and enabling the AutoMRE function to allow the program to automatically determine sufficient bootstrapping number. We conducted ML analyses using three partitioning schemes on the concatenated dataset: unpartitioned, locus-partitioned (partitioned by UCE locus), and rclusterf-partitioned using PartitionFinder2 (475 partitions; Lanfear et al., 2017). We used the GTR+Γ model of molecular evolution for all inferences including the rclusterf-partitioned analysis. The rclusterf partitioning scheme was favored through AICc analysis, thus we used the rclusterf-partitioned RAxML tree for downstream analyses (Supplementary Table S3.3).

For the BI analyses, we conducted an inference using the rclusterf-partitioned matrix in ExaBayes v1.5 (Aberer et al., 2014) on the CIPRES computing cluster. We conducted two runs with 1 million generations, a checkpoint interval of 10,000, four coupled chains, and a 10% burn-in, with all other setting left at the default. We confirmed convergence and adequate effective sample size (ESS) values of both runs combined using Tracer v1.7.1 (Rambaut et al., 2018). We also enabled the "consense" function to produce an output consensus tree with a threshold of 50% and a burn-in of 10%.

We conducted GT analyses using IQ-TREE v1.6.12 (Nguyen et al., 2015) and ASTRAL-III v5.6.3 (Zhang et al., 2018) on the Midway2 computing cluster. We implemented fast ML inferences in IQ-TREE for each UCE locus, including 1000 bootstraps, and exported the inferred tree, with labelled bootstrap support, for each locus. Previous work has shown that statistically binning loci into clusters can reduce error from loci with low information content (Branstetter et al., 2017), and thus we implemented the statistical binning pipeline (Mirarab et al., 2014). However, the binning method failed to bin genes together, even with a threshold set to 90%, due to the presence of highly-supported conflicts (pers. comm. S. Mirarab), so we proceeded without binning UCE loci. We ran ASTRAL-III on our ML tree set, after collapsing branches with <10% support to improve accuracy (Zhang et al., 2017), using local posterior probability (localPP) to assess node support.

3.3.4 Divergence dating

We implemented divergence dating analyses on our rclusterf-partitioned RAxML tree in MCMCTree, which is part of PAML v4.9e (Yang, 2007). Due to current computational limitations, phylogenomic studies typically use a small subset of the entire available data matrix when conducting divergence dating in programs like

BEAST2 (Bouckaert et al., 2019). We chose MCMCTree as it is a more efficient program and also now has a sister R package, MCMCtreeR (Puttick, 2019), for setting and visualizing intuitive node priors based on fossil data, a former limitation. Furthermore, we used the "approximate likelihood" method in MCMCTree to calculate the likelihood function during MCMC iteration (Reis & Yang, 2011), allowing the use of all 1,300 UCE loci in our 70% taxon complete dataset. We set uniform node age priors on outgroup nodes and the *Polyrhachis* crown node in MCMCtreeR, based on the means and confidence intervals from previous phylogenetic studies (Moreau & Bell, 2013; Blaimer et al., 2015; Mezger & Moreau, 2016; Supplementary Table S3.4). We used the HKY + $\Gamma 4$ DNA substitution model (with Gamma shape parameter $\alpha = 0.5$) and an independent rates relaxed clock model, which exhibits consistently better performance compared to the correlated rates relaxed clock model and better performance compared to the strict clock model when rate variation is medium or high (Brown & Yang, 2011). After initial test runs, we also adjusted the "rgene_gamma" ($\alpha = 2$, $\beta = 30$, αD = 1) and "sigma2_gamma" ($\alpha = 2$, $\beta = 1$, αD = 1) priors to values with means close to initial run values for these parameters. In order to ensure proper MCMC convergence, we conducted 5 independent runs of 2 million generations with 20,000 samples and combined these runs into one MCMC file, for a combined total of 10 million generations and 100,000 samples in the posterior. We used Tracer to assess convergence and ESS values after manually removing the first 10% from each independent MCMC run as burn-in, and also assessed the impact of our DNA data relative to the priors on our posterior tree inference by implementing priors-only runs of MCMCTree with the same settings described above except with our DNA dataset excluded. We used the posterior tree with RAxML-inferred topology and mean node ages from the 95% HPD distribution inferred in MCMCTree for downstream macroevolutionary analyses.

3.3.5 Trait database

We collected morphological and geographic range trait data for the 160 *Polyrhachis* species included in our dated MCMCTree phylogeny. To measure linear measurements of worker body size and total spine length, we downloaded up to three available image sets from AntWeb (antweb.org; accessed December, 2019) and AntWiki (antwiki.org; accessed December, 2019), where each image set includes one lateral view and one dorsal view for a sample. Images were available for nearly all species, including 81 species (50.6%) with three image sets or more, 46 species (28.8%) with two image sets, and 27 species (16.9%) with 1 image set, while only 6 species (3.8%) had no image sets available. From these images, we conducted linear measurements in ImageJ v2.0.0 (Schindelin et al., 2012; Rueden et al., 2017). To capture the "Body Size" metric, we used Weber's Length, a standard proxy metric of body size used in ants (diagonal length of the mesosoma in lateral view; Supplementary Fig. C.1). To capture total spine length ("Spine Length"), as we were interested in overall tissue investment in spine production, we measured the linear distance from the base of a spine to the tip, for one of each pair of spines present on the pronotum, mesonotum, propodeum, and petiole, and summed these values together. For each spine in a sample, we used the image orientation (lateral or dorsal) that was most parallel to the plane of projection of the spine. For a few cases, a singular spine – or spine triplet – was present (e.g. in *P. (Myrmothrinax)* spp.) rather than a typical pair of spines. In this situation, we included half the length of the solitary spine in our total spine length measurement (Supplementary Table S3.1) in order to correspond to our measurement of spine pairs, which only includes one (i.e. 50%) of the total spine production for that pair. For curved spines, we used the "segmented line" tool to measure linear length along the curvature of the spine. See Supplementary Fig. C.1 for examples of different spine measurements. We also used "Relative Spine

Length" (Spine Length/Body Size) for some analyses. Measurements for species with multiple image sets were averaged together.

We collected geographic range data using AntMaps.org (Guénard et al., 2017; Janicki et al., 2016), an online database of ant distribution records. For each of the 160 species in our dataset, we recorded two metrics: (1) presence/absence within three large biogeographic categories, "Afrotropical", "Oriental", and "Australasian" (Lomolino et al., 2005; Mezger & Moreau, 2016), using Wallace's Line (Wallace, 1863) to separate "Oriental" from "Australasian"; and (2) the number of subdivided regions in AntMaps.org in which a species has been collected. Although the divisions in the database are geopolitical based on country boundaries, large countries like China, India, Australia, and Indonesia are subdivided into states and similar subdivisions. Thus, this metric, which we term "Range Size" (ranging from 1 to 51 in our data), should provide an adequate approximation of geographic range at a finer scale than the three large biogeographic regions.

3.3.6 Macroevolutionary analyses

We inferred ancestral states along our time-scaled phylogeny for Relative Spine Length, Body Size, and Range Size using the "contMap" function in phytools v0.6 (Revell, 2012; Revell, 2013) implemented in RStudio v1.2.5033 (RStudio Team, 2015; R Core Team, 2018). We also inferred ancestral geographic ranges using BioGeoBEARS v1.1.2 (Matzke, 2013). We utilized the "DEC" model without the jump dispersal/founder-event speciation parameter ("J"), given the very large size of our three biogeographic regions and concerns pertaining to the DEC+J model (Ree & Sanmartín, 2018).

We conducted diversification analyses using several methods. To assess shifts in diversification independent of *a priori* trait information, we used BAMM v2.5.0 and

BAMMtools v2.1.7 (Rabosky 2014; Rabosky et al., 2014). We enabled the "sampleProbsFilename" option to account for differing levels of taxon sampling across the 12 *Polyrhachis* subgenera included in our phylogeny. We set values for the "expectedNumberOfShifts", "lambdaInitPrior", "lambdaShiftPrior", and "muInitPrior" priors using the "setBAMMpriors" function in BAMMtools, which is designed to select appropriate priors based on features of the input dataset. With all other settings left at the default, we ran the MCMC for 10 million generations, sampling every 10,000 generations, and assessed convergence in Tracer. We then evaluated the event data using BAMMtools, to assess the inferred average phylorate across the *Polyrhachis* phylogeny, as well as the 95% Credible Set of distinct shift configurations (i.e. the set of distinct shift configurations that account for 95% of the probability of the data).

To assess evidence for trait-based diversification, we implemented an analysis in QuaSSE (FitzJohn, 2010) in the package diversitree v09-13 (FitzJohn, 2012). For each trait (Spine Length, Relative Spine Length, Body Size, and Range Size), we used the "starting.point.quasse" function to set starting points for each model based on parameters estimated using birth-death and Brownian motion models of evolution. We then used an ANOVA to compare the fit of a trait-correlated versus trait-uncorrelated model of diversification. Due to documented Type 1 error issues with the SSE suite of models under some scenarios (Rabosky & Goldberg, 2015), we also used *ES-sim* v1.1 (Harvey & Rabosky, 2018), which tests for correlations between summary statistics of phylogenetic branching patterns and trait variation at the tips of a phylogeny and is designed as an alternative to QuaSSE. We ran 1000 simulations in *ES-sim* for each trait.

Finally, to test for correlations between Spine Length, Body Length, and Range Size, we conducted a phylogenetic generalized least squares (PGLS) analysis in the

R package caper v1.0.1 (Orme, 2018). One of our predictions was that spine length should promote, and thus be positively correlated with, increased geographic range size, thus we set Range Size as the response variable and included both Spine Length and Body Size as factors. We also tested for correlations between Body Size and Spine Length to test for potential allometric scaling between Spine Length and Body Size. All variables were log-transformed prior to analysis (Mundry, 2014), and we coestimated Pagel's lambda for all PGLS analayses (Revell, 2010).

3.4 Results

3.4.1 UCE capture statistics

Our 70% taxon complete matrix includes 161 samples from 160 *Polyrhachis* species, representing approximately 23% of the hyperdiverse genus (antcat.org; accessed February, 2020), and 15 outgroup Formicine taxa. The raw sequence data consisted of an average of 7.2 million reads per sample with an average length of 140 bp (Supplementary Table S3.5). The Trinity assembly step resulted in an average of 98,505 contigs with a mean length of 377.7 bp and average coverage of 5.7X. After the finding UCE loci step, we recovered an average of 1,653 UCE contigs per sample, with a mean contig length of 865 bp (Supplementary Table S3.5). The final matrix of concatenated UCE loci was 1,038,583 bp long and included 1,300 UCE loci alignments and 174,117 informative sites.

3.4.2 Phylogenomic analyses

Our PartitionFinder2 analyses, using the rclusterf algorithm, produced a partitioning scheme with 475 subsets. The rclusterf-partitioned dataset was favored via AICc analysis, although the mean branch support, tree length, mean branch length, and branch length variance were relatively similar for all partitioning schemes (Supplementary Table S3.3), suggesting minimal impacts of partitioning scheme.

The final tree topologies for our maximum likelihood (ML), Bayesian inference (BI), and gene-tree (GT) analyses were largely consistent with each other. The ML and BI topologies were identical to each other, with only some differences in bootstrap versus posterior probability node support (Fig. 3.1, Supplementary Figs. C.2, C.3). The GT topology was similar to that of the ML and BI trees at the subgeneric level, with some exceptions at the species level (Supplementary Fig. C.4). The relationships between species within the *P. (Cyrtomyrma)* subgenus differed from the ML/BI topology, as do a few relationships within other subgenera. The most unusual incongruity in the GT inference is the placement of *P. lamellidens*, a well-supported member of the highly distinct *P. (Polyrhachis)* subgenus. In the GT topology, this species is placed away from the *P. (Polyrhachis)* subgenus and instead sister to the entire *Polyrhachis-Myrma* clade. This major inconsistency led us to favor the ML/BI topology, which we used for divergence dating. The subgeneric relationships found in our ML/BI tree topology are also broadly similar to the relationships reported in Mezger and Moreau (2016), with a couple notable exceptions. Mezger and Moreau found *P. (Myrmhopla)* to be polyphyletic with five distinct clades, whereas we infer less polyphyly with only four distinct clades. We do, however, find high support for *P. (Myrmatopa)* as polyphyletic with two distinct clades, where Mezger and Moreau inferred monophyly. Notably, our phylogeny includes twice as many described species and significantly more genetic information than the former phylogeny (see Supplementary Information C for more discussion on comparisons between these two studies). Overall, the consistency of our trees across differing phylogenetic inference methods, and similarities with Mezger and Moreau (2016), supports the robustness of our topological inference, with the gene-tree method producing the most divergent results.

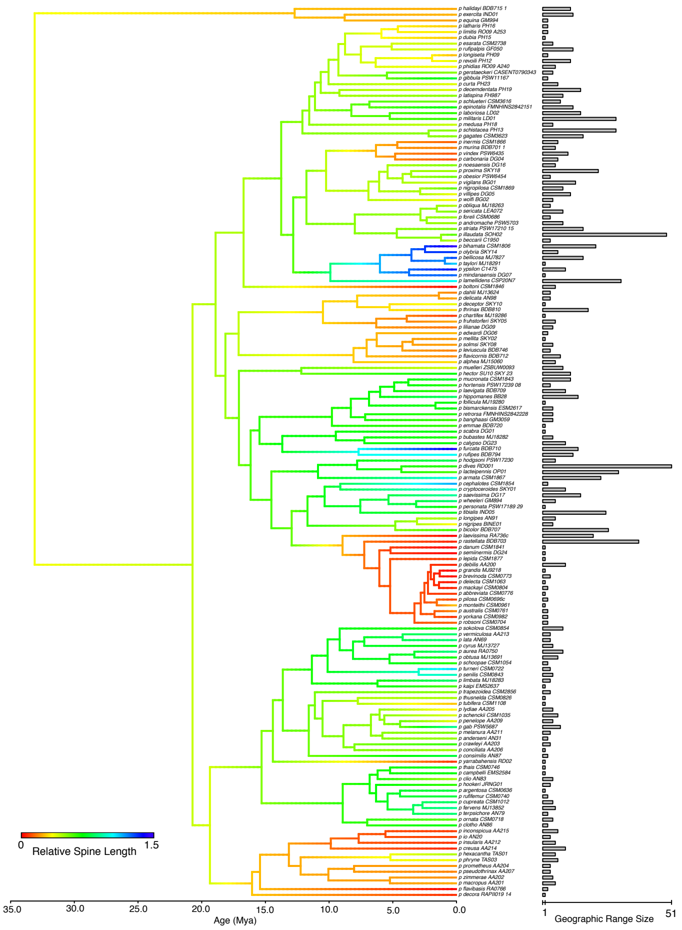


Figure 3.1: MCMCTree time-scaled phylogeny based on RAxML topology. Bars display geographic range size for each species, and the ancestral state reconstruction of Relative Spine Length is mapped along the phylogeny.

Divergence dating in MCMCTree using our rclusterf-partitioned RAxML starting tree reconstructed a node age for crown *Polyrhachis* of 33 Mya (95% HPD: 21.16-48.73 Mya), a slightly younger age compared to the previous molecular phylogenetic study of the genus that inferred a crown age of 42 Mya (Mezger & Moreau, 2016). Because we designated outgroups in our RAxML analysis, the outgroup relationships are not resolved with respect to *Polyrhachis*, but our inferred age of the crown Formicinae subfamily, 126 Mya (95% HPD: 111.5-138.76 Mya), is consistent with a previous phylogenomic study of Formicinae (Blaimer et al. 2015) while older than the age inferred by a family-wide phylogenetic study of the ants (Moreau & Bell, 2013; Nelsen et al. 2018). Diagnostic priors-only runs in MCMCTree established that our data substantially impact both inferred node dates and estimated relative branch lengths between subclades (Supplementary Figs. C.5-C.7). Outgroups were pruned for subsequent analyses using our time-scaled MCMCTree phylogeny.

3.4.3 Macroevolutionary analyses

Our database includes morphological trait data for 154 of the 160 *Polyrhachis* species in our 161-tip MCMCTree phylogeny (Supplementary Table S3.1). In addition to outgroups, we pruned the six taxa without morphological information as well as one of the two *P. armata* tips to ensure even sampling of species included in our tree. This 154-tip *Polyrhachis* phylogeny was used for all macroevolutionary analyses.

Ancestral state estimation analyses demonstrate wide variation in relative spine length, body size, and range size, with multiple phylogenetically independent gains and losses of trait states for all three of these traits (Fig. 3.1, Supplementary Figs. C.8-C.10). Very long relative spine lengths are inferred to have evolved independently at least five times, while very small spines - or complete spine loss - has independently occured at least seven times (Supplementary Fig. C.8). Large body size appears to have evolved at least ten times and small body size at least five times (Supplementary Fig. C.9). One portion of the *Polyrhachis* phylogeny exhibits consistenty small range sizes (species found in approximately one to three regions), wheras the rest of the tree is more variable, including at least three independent gains of very large range size (Supplementary Fig. C.10). As in Mezger and Moreau (2016), a joint Oriental/Australasian origin is inferred as the ancestral state of crown *Polyrhachis* in our BioGeoBears analysis, consistent with a Southeast Asian origin of the genus (Supplementary Fig. C.11). Relative to our ancestral range estimation results based on three broad biogeographic categories, our finer-scale range size data reveals fairly high evolutionary lability of range size, with sizes ranging from a small distribution of one territory up to very large distributions of 51 territories (Fig. 3.2, Supplementary Fig. C.10).

Diversification analyses, implemented using three different methods, consistently found no association between diversification rate and any of our traits (spine length, body size, and range size). While our BAMM analyses produces an average phylorate plot with an apparent rate increase in the *Cyrtomyrma* subgenus – which is notable for a nearly complete lack of spines – the majority of configurations in the 95% Credible Shift Set (0.52 frequency) lack an inferred rate shift in the subgenus (Fig. 3.2). Furthermore, the single most frequent configuration (0.39 frequency) infers zero rate shifts. In /textitES-sim, we recovered no association between diversification and spine length ($P = 0.829$), relative spine length ($P = 0.759$), body size ($P = 0.454$), or range size ($P = 0.524$) (Supplementary Table S3.6). Similarly, our QuaSSE analyses did not support a trait-dependent model of diversification when compared to a trait-independent model, for spine length ($P = 0.646$), relative spine length ($P = 0.852$), body size ($P = 0.398$), or range size ($P = 0.771$) (Supplementary Table S3.6).

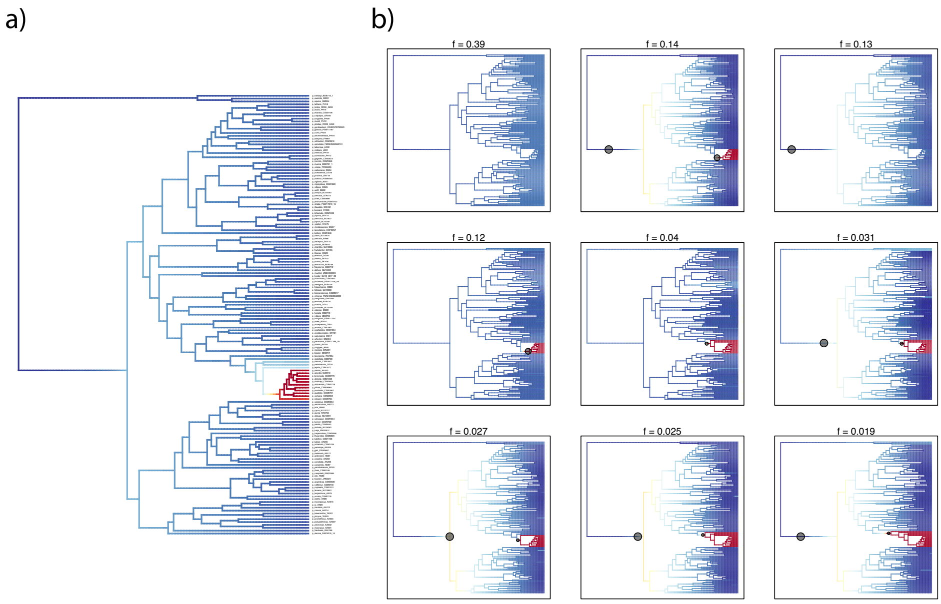


Figure 3.2: Results from a BAMM analyses of the time-scaled MCMCTree phylogeny. (a) phylorate plot. (b) 95% credible shift set (nine plots representing 0.922 frequency displayed).

PGLS analyses revealed some evolutionary correlations between our log-transformed traits. In particular, we found a significant positive association between spine length and geographic range size ($P = 0.026$, $R^2 = 0.037$), while finding that body size is not associated with range size ($P = 0.857$) (Fig. 3.3, Table 3.1). Spine length also positively scales with body size ($P = 0.002$, $R^2 = 0.06$), without clear evidence of allometric scaling (Fig. 3.3, Table 3.1).

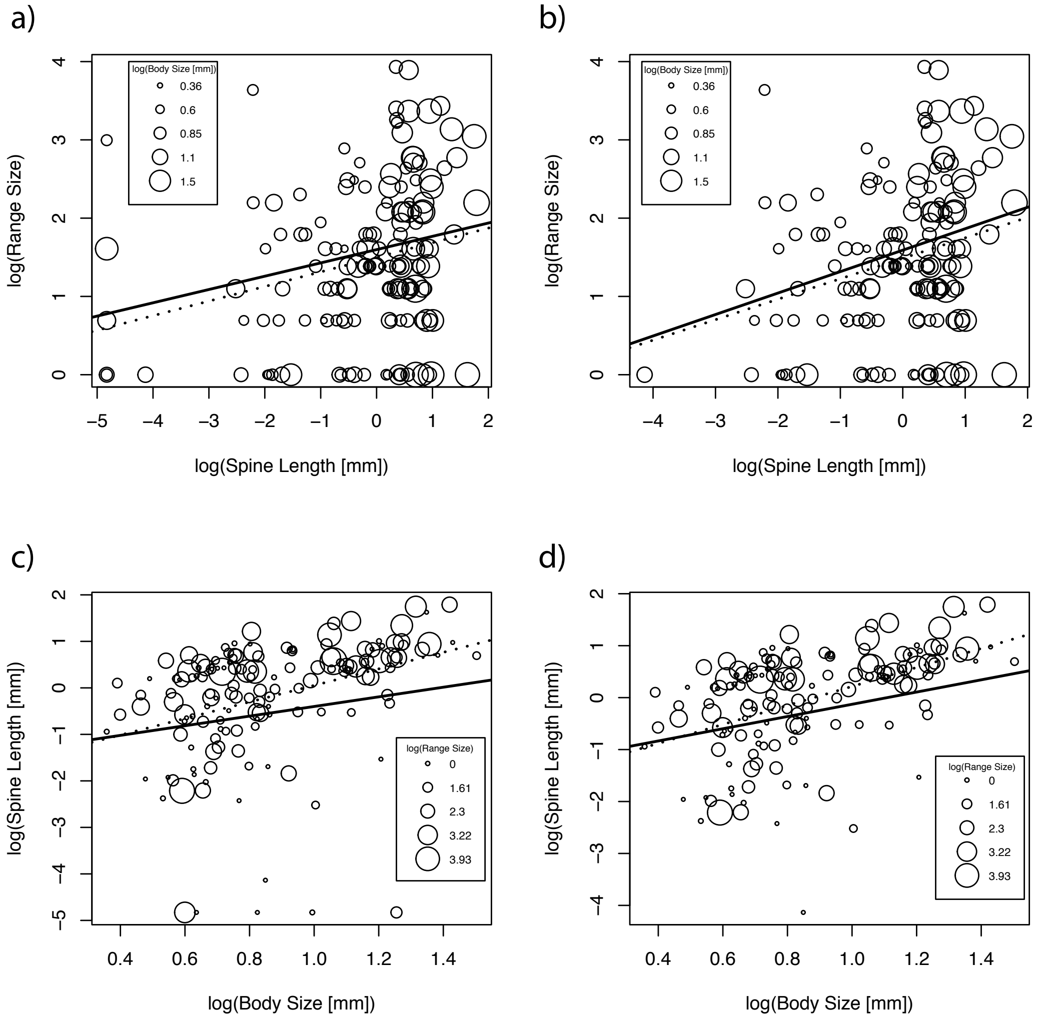


Figure 3.3: Phylogenetic generalized least squares (PGLS) analyses including Spine Length, Body Size, and Range Size. (a) and (b) display results from a bivariate PGLS model including Spine Length as a predictor of Range Size, with and without zero-spine taxa included, respectively. Point size corresponds to Body Size. (c) and (d) display results from a bivariate PGLS model including Body Size as a predictor of Spine Length, with and without zero-spine taxa included, respectively. Point size corresponds to Range Size. In (a)-(d), the solid trend line corresponds to the bivariate PGLS analysis, and the dotted trend line corresponds to a bivariate standard regression conducted using the "lm" function in R. The values for all variables have been log-transformed. See Table 1 for full results from the multivariate PGLS analyses including both Spine Length and Body Size as predictors of Range Size.

Table 3.1: Results from phylogenetic generalized least squares (PGLS) analyses including Spine Length and Body Size as predictors of Range Size. We conducted two multivariate PGLS analyses – one with zero-spine taxa included, and one with zero-spine taxa excluded.

All taxa included	*Estimate*	*Std. Error*	*t value*	*p value*
(Intercept)	1.541	0.491	3.137	**0.002**
log(Spine Length)	0.166	0.074	2.242	**0.026**
log(Body Size)	0.063	0.347	0.181	0.857
Zero-spine taxa excluded				
(Intercept)	1.677	0.486	3.451	**<0.001**
log(Spine Length)	0.286	0.286	0.107	**2.689**
log(Body Size)	-0.095	0.369	-0.258	0.797

3.5 Discussion

We reconstructed a large phylogeny of the hyperdiverse spiny ant genus *Polyrhachis* using a phylogenomic UCE approach, resulting in a robust time-scaled tree that includes 160 species from 12 of the 13 recognized subgenera in the genus. Using this phylogeny and our compiled database of spine length, body size, and geographic range size data, we found a positive correlation between spine length and geographic range (Fig. 3.1, Fig. 3.3). Although the R^2 value is low, this result supports the hypothesis that spines contribute to broader geographic range sizes in /textitPolyrhachis, potentially due to a reduction in worker loss from predation. Interestingly, our results also consistently support no association between spine length and diversification, regardless of analysis used (Fig. 3.2, Supplementary Table S3.6), in contrast to our expectation that spines should promote elevated diversification rates.

While we find a positive association between defensive traits and geographic range, some studies in plants find the reverse: defense specialization adapted to local enemies narrows geographic range and/or constrains range evolution (Agrawal et al., 2005; Siemens et al., 2009). Our alternative result may arise from the fact that ant spines should serve as quite generalized and also fairly cheap defenses against most myrmecophagous vertebrates, and thus do not carry the spatially constraining costs associated with specialization. Thus, similar to invasive species experiencing enemy release that facilitates invasion into novel environments (Keane & Crawley, 2002), long spines in *Polyrhachis* workers may afford escape from widespread predators – such as frogs (Ito et al., 2016) and spiders (Blanchard et al. *in press*) – regardless of geographic location. Direct tests of a defensive trait-geographic range size relationship are surprisingly rare, but Luiz et al. (2013) present a similar case in tropical reef fishes, where adult defensive traits (schooling behavior and nocturnal activity) predicted larger geographic range size.

The lack of a relationship between spines and diversification in *Polyrhachis* is intriguing, as it contrasts with previous work across all ants at the taxonomic scale of the genus (Blanchard & Moreau, 2017). It is possible that geographic range expansion in single species promotes increased gene flow (Bohonak, 1999), such that more spinescent clades speciate at slower rates than less spinescent clades. Some studies on bird evolution support this dynamic, where increased dispersal abilities suppress diversification rates (Claramunt et al., 2012; Weeks and Claramunt, 2014). Spine expression may also carry costs that are not detectable in this study, e.g. restriction of nesting site location (Wilson, 1959), that negate any enhancement of diversification rate resulting from expanded geographic range size. Notably, while our phylogenetic inference represents twice as many described species as the previous *Polyrhachis* phylogeny (Mezger & Moreau, 2016), our tree still includes only 22% of all described taxa in the genus. Furthermore, among the three diversification analyses, only BAMM was able to incorporate variable taxonomic sampling information, and even this correction is not a substitute for real data. Thus, although all three analyses, which are each fundamentally different in their approach, were concordant in failing to support spine-based diversification in *Polyrhachis*, further phylogenetic sampling is necessary to more robustly establish the relationship between cuticular spines and diversification in the spiny ants.

Overall, our study provides a relatively rare investigation directly assessing the role of a defensive trait on geographic range size, and ultimately supports the hypothesis that defensive spines are a factor in increased range size in *Polyrhachis* ants. Additionally, neither spine length nor range size expansion appear to be associated with elevated diversification rates, a dynamic that should be the focus of future research. Our work adds to the growing body of evidence that defensive traits – and especially spines – play a key role in the evolution of ants and may significantly contribute to the evolutionary persistence and global dominance of this hyperdiverse clade of insects.

4 Supplementary Figures, Chapter 1

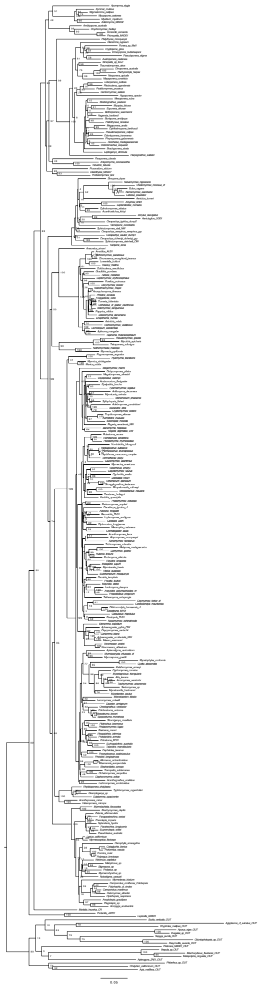


Figure 4.1: Maximum likelihood tree inferred in RAxML, including 648 in-group species and 18 out-group species. Nodes are labeled with bootstrap support values and branch lengths are proportional to substitution/site as indicated by the bottom legend inset.

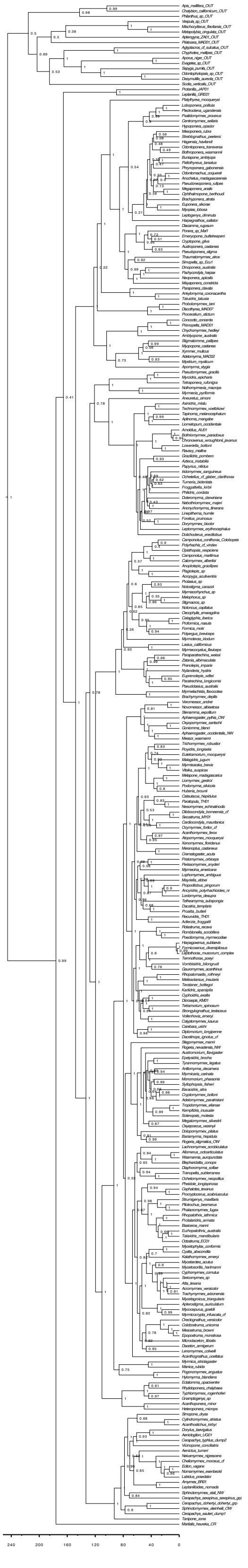


Figure 4.2: Dated Bayesian maximum clade credibility (MCC) tree, including 275 in-group and 16 out-group species. Nodes are labeled with posterior probability (PP) values. Scale bar is in millions of years before present.

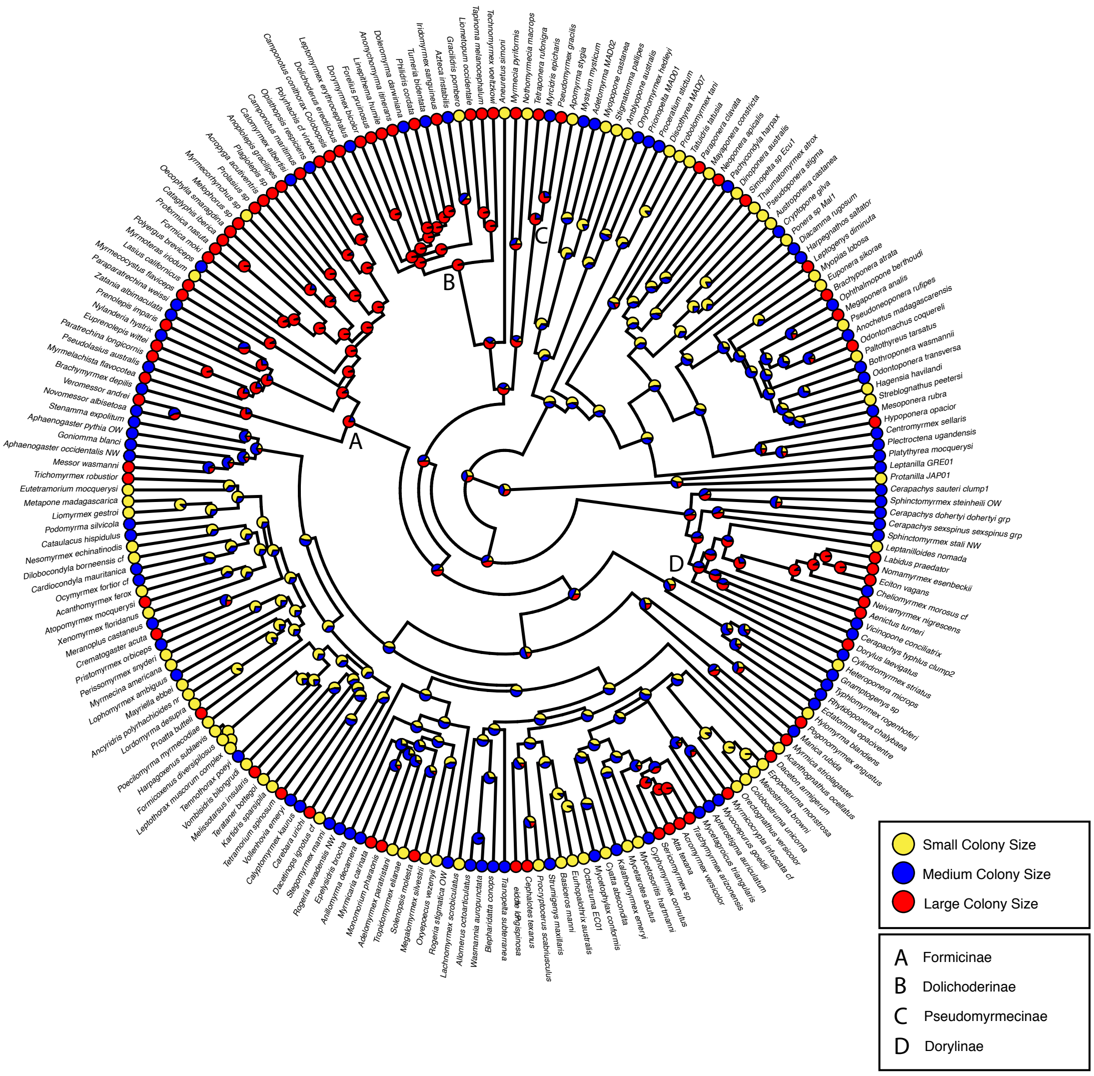


Figure 4.3: Ancestral state reconstruction (stochastic character mapping) for colony size. The crown nodes for subfamilies noted in the main text are also labeled A-D.

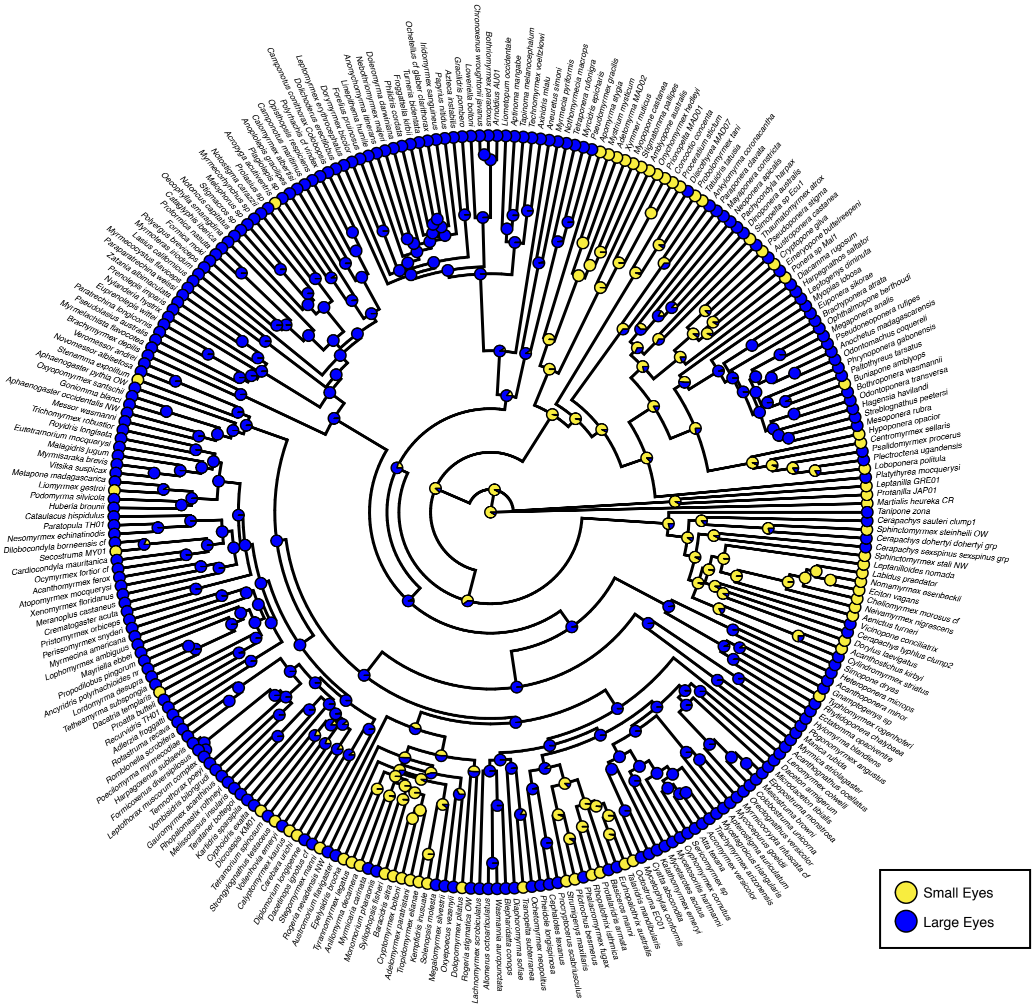


Figure 4.4: Ancestral state reconstruction (stochastic character mapping) for eye size.

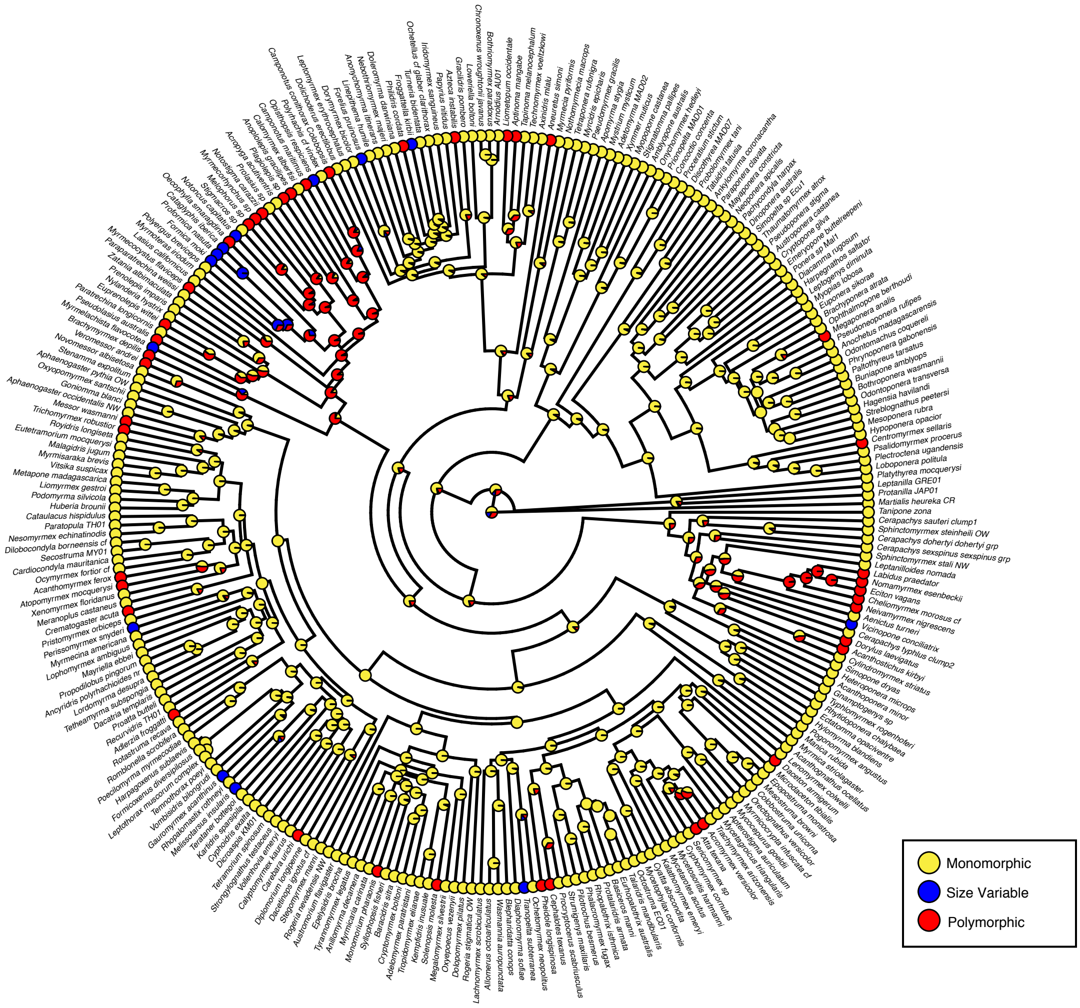


Figure 4.5: Ancestral state reconstruction (stochastic character mapping) for polymorphism.

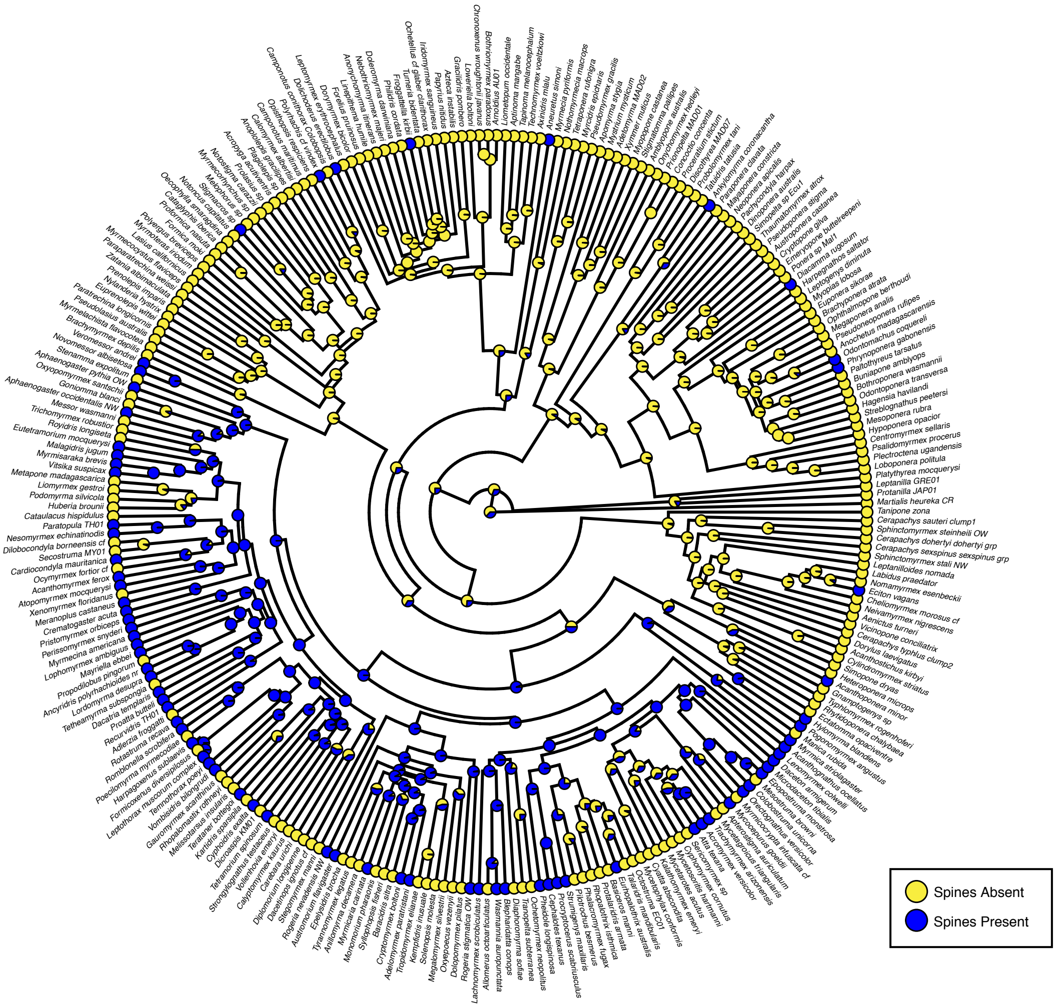


Figure 4.6: Ancestral state reconstruction (stochastic character mapping) for spines.

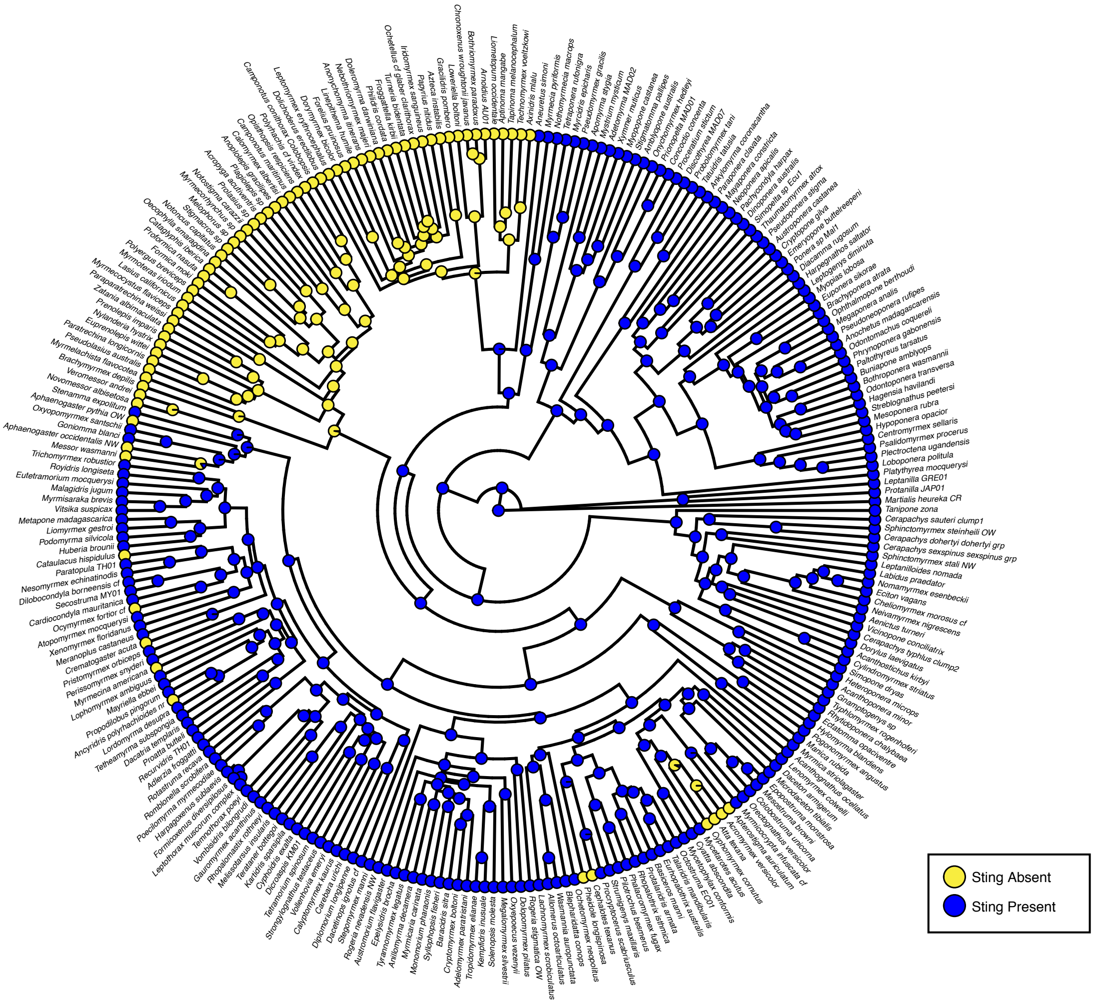


Figure 4.7: Ancestral state reconstruction (stochastic character mapping) for sting.

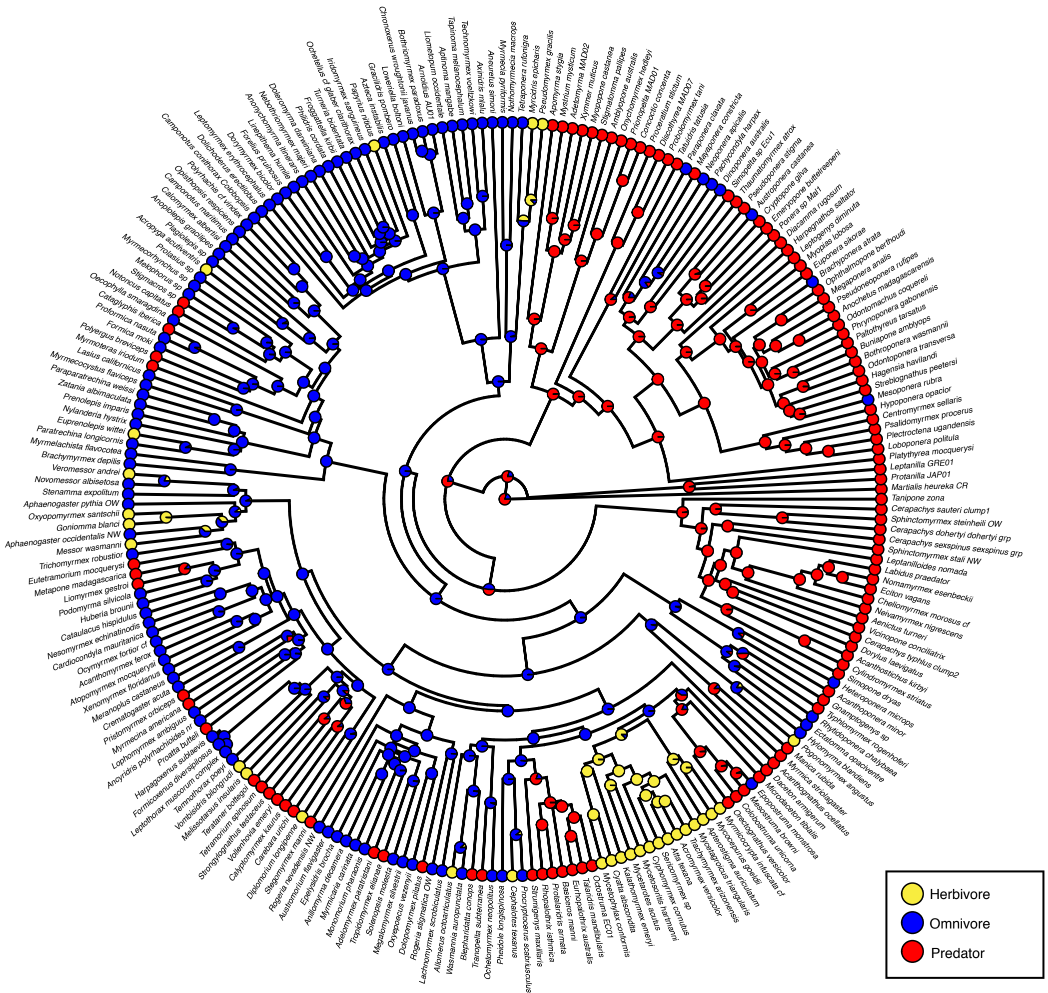


Figure 4.8: Ancestral state reconstruction (stochastic character mapping) for diet.

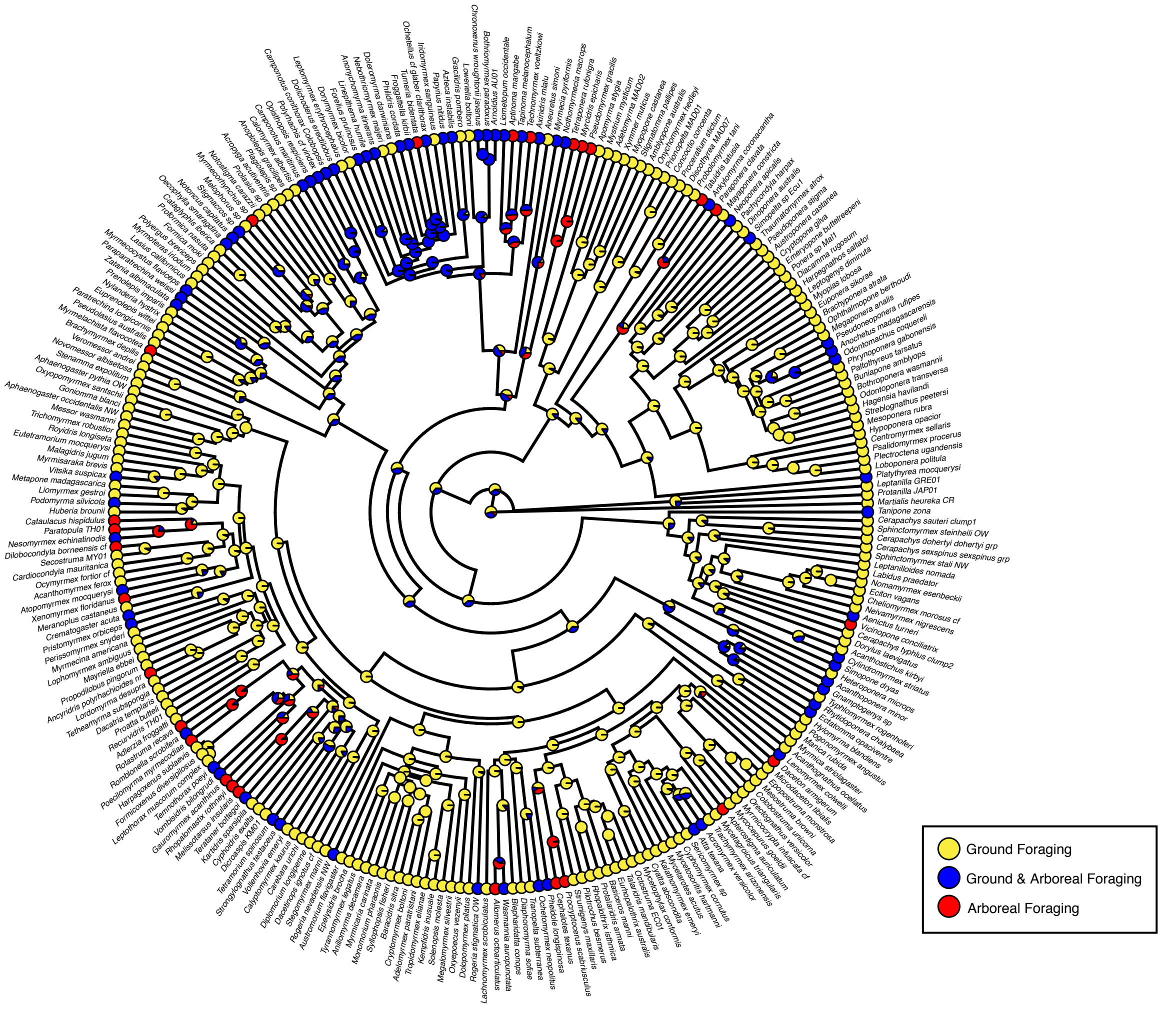


Figure 4.9: Ancestral state reconstruction (stochastic character mapping) for foraging location.

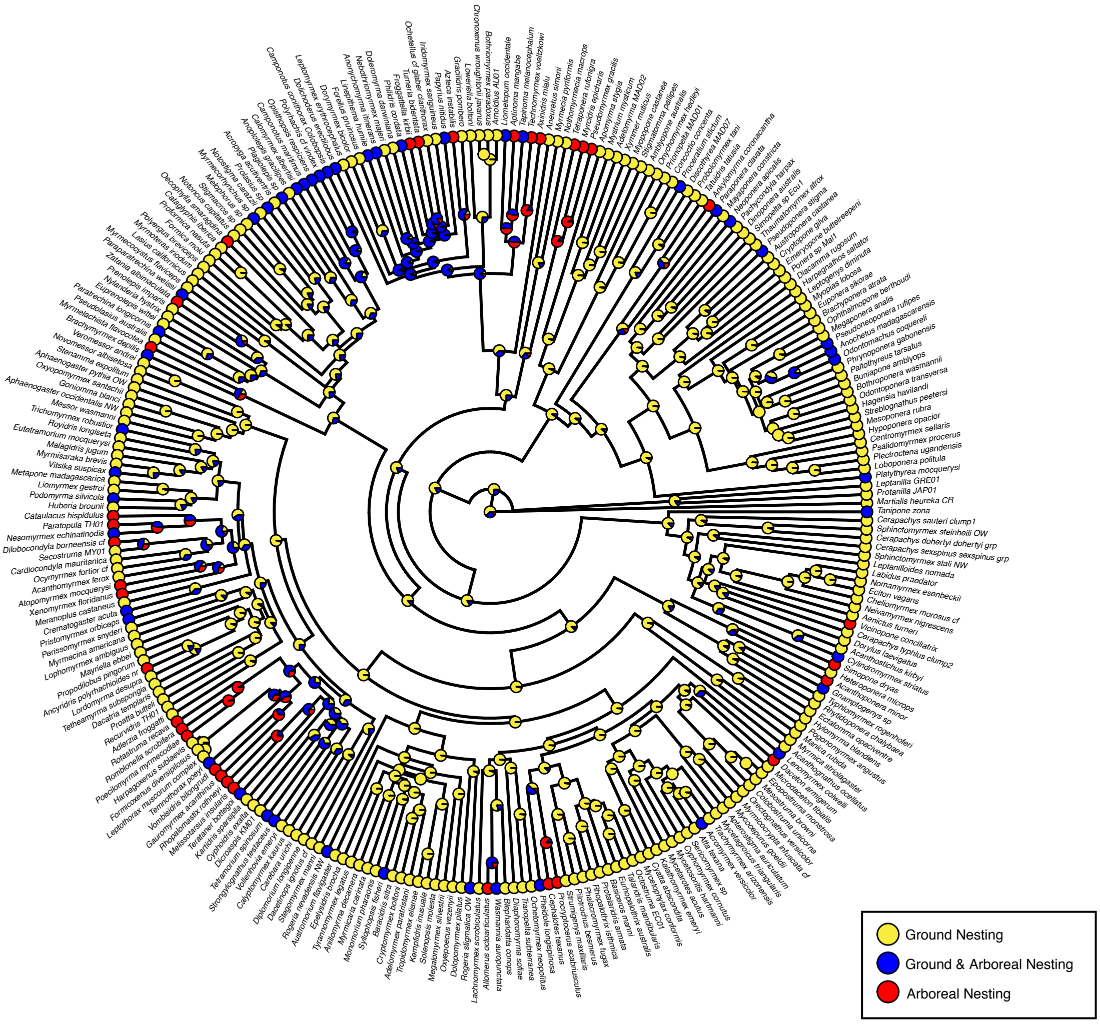


Figure 4.10: Ancestral state reconstruction (stochastic character mapping) for nesting location.

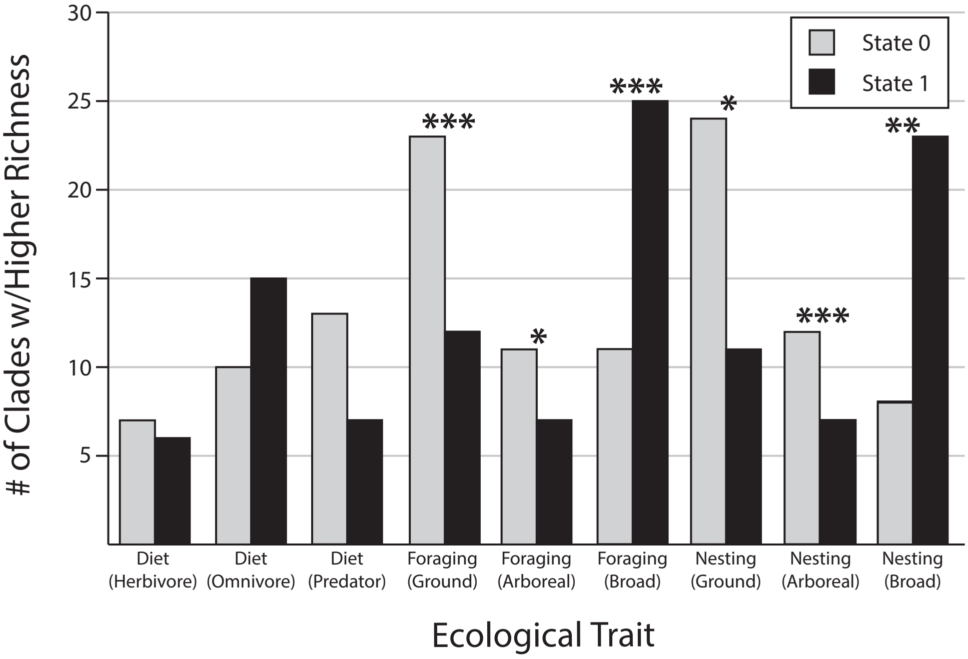


Figure 4.11: Results of sister clade analyses of ecological traits and diversification. An association between diversification and trait states (0 or 1) is assessed using a model-based likelihood ratio test (Paradis 2011; *$P < 0.05$, **$P < 0.01$, ***$P<0.001$).

5 Supplementary Figures, Chapter 2

Figure 5.1: Experimental design for resource discovery trials. Image credit: B.D. Blanchard.

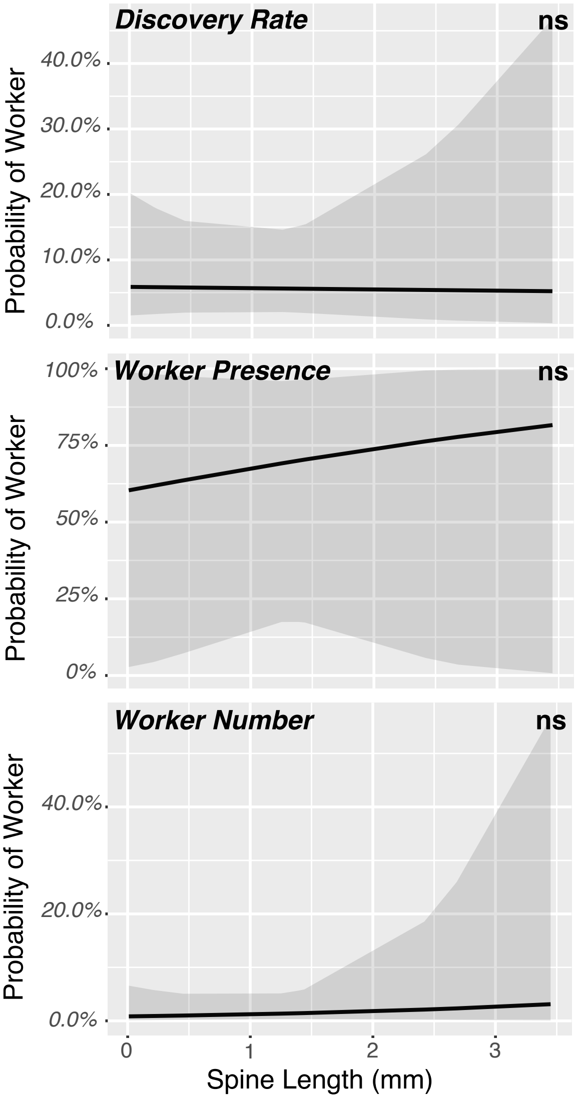


Figure 5.2: Resource discovery results for the multispecies (n = 11) trials with *P. (Cyrtomyrma)* species (n = 2) included. ns = not significant ($P > 0.1$). Grey zones indicate the boundaries of the 95% prediction interval, i.e. the interval expected to contain 95% of future observations for a given spine length.

6 Supplementary Figures, Chapter 3

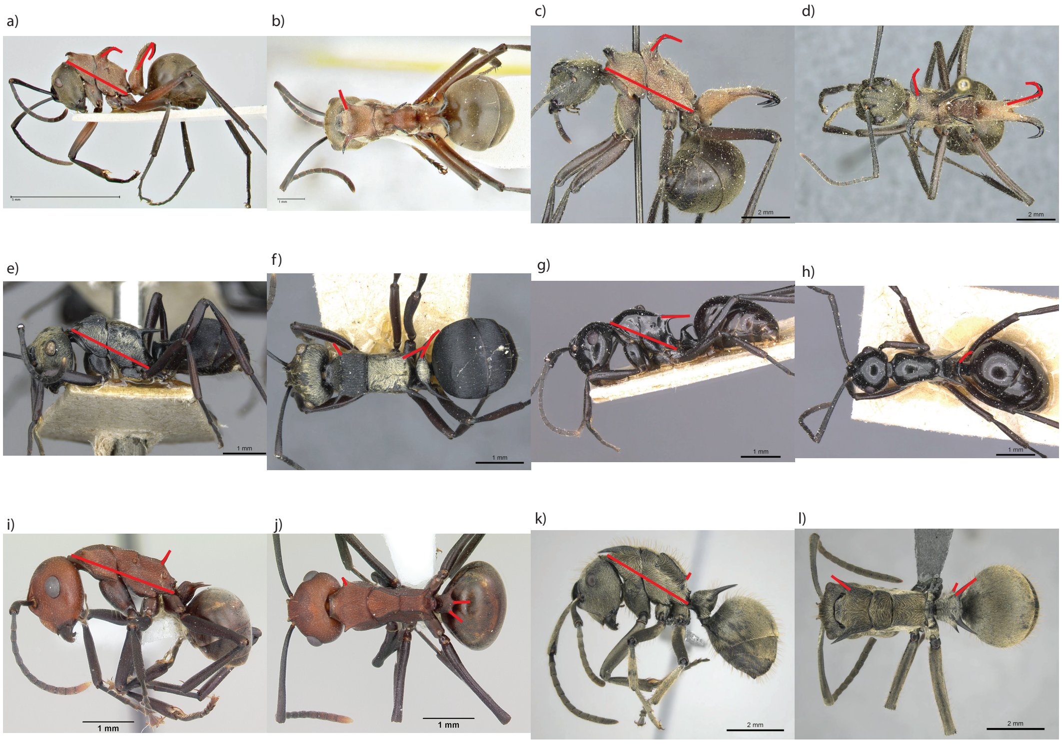


Figure 6.1: Examples of spine measurements. Red lines show the path traced using the "Straight Line" or "Segmented Line" tools in ImageJ. Species (and photo credit), from top left: (a-b) *P. bellicosa* (Christiana Klingenberg), (c-d) *P. ypsilon* (Zach Lieberman), (e-f) *P. argentosa* (Zach Lieberman), (g-h) *P. emmae* (Will Ericson), (i-j) *P. deceptor* (April Nobile), (k-l) *P. epinotalis* (Bradley Reynolds). From www.antweb.org under a Creative Commons Attribution License. Accessed 21 December 2019.

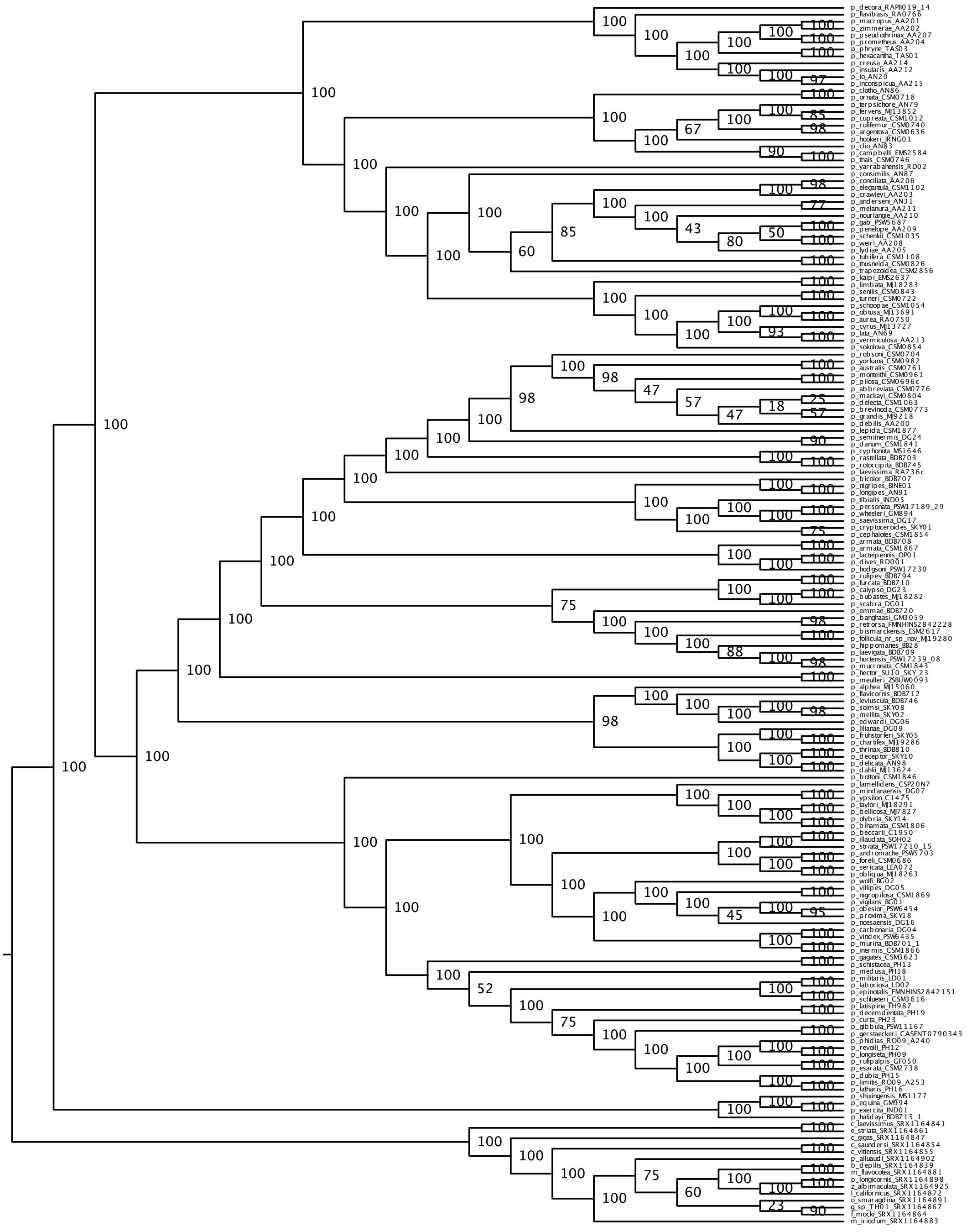


Figure 6.2: *Polyrhachis* phylogenetic topology inferred with the rclusterf-partitioned dataset in RAxML.

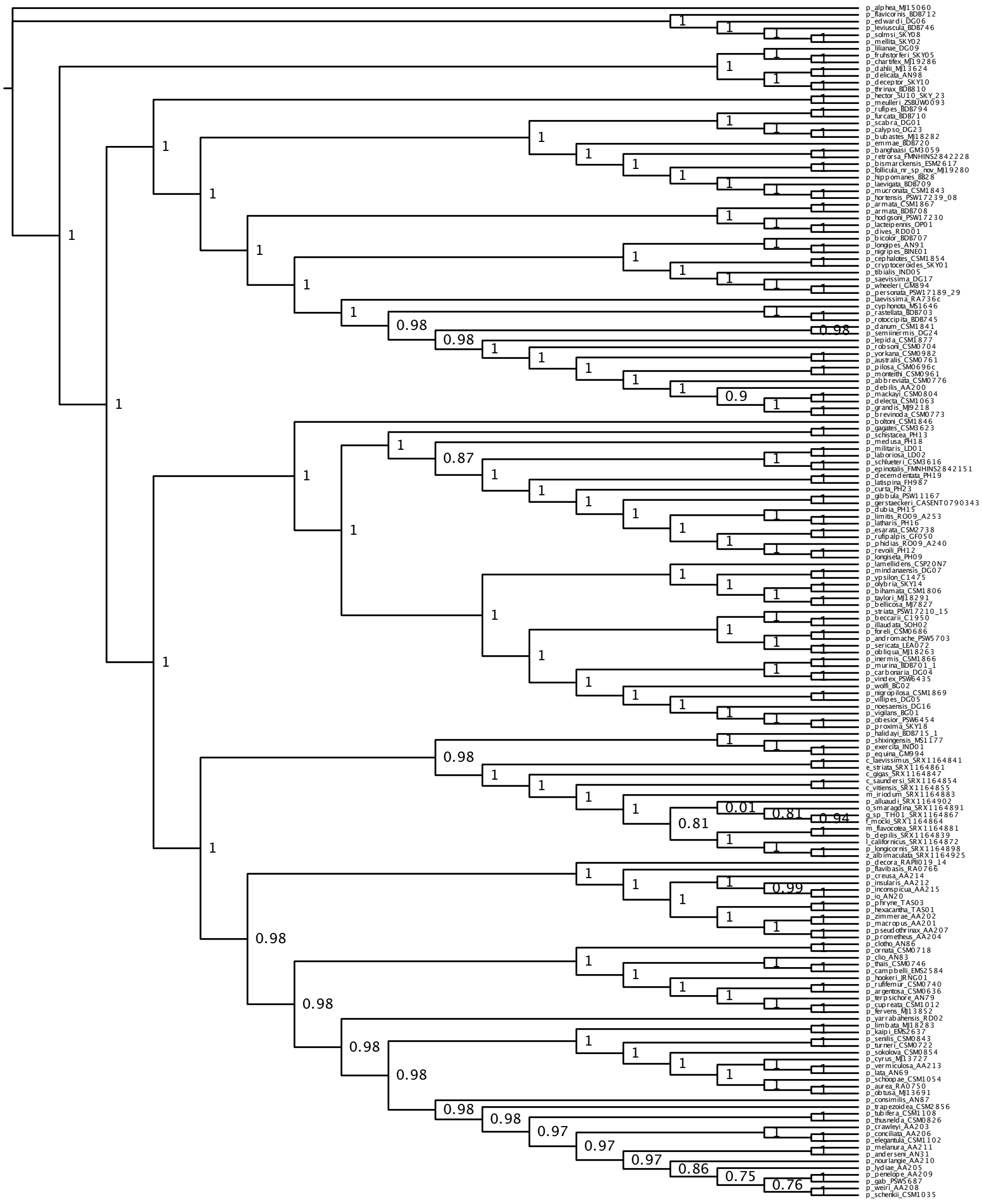


Figure 6.3: *Polyrhachis* phylogenetic topology inferred using ExaBayes.

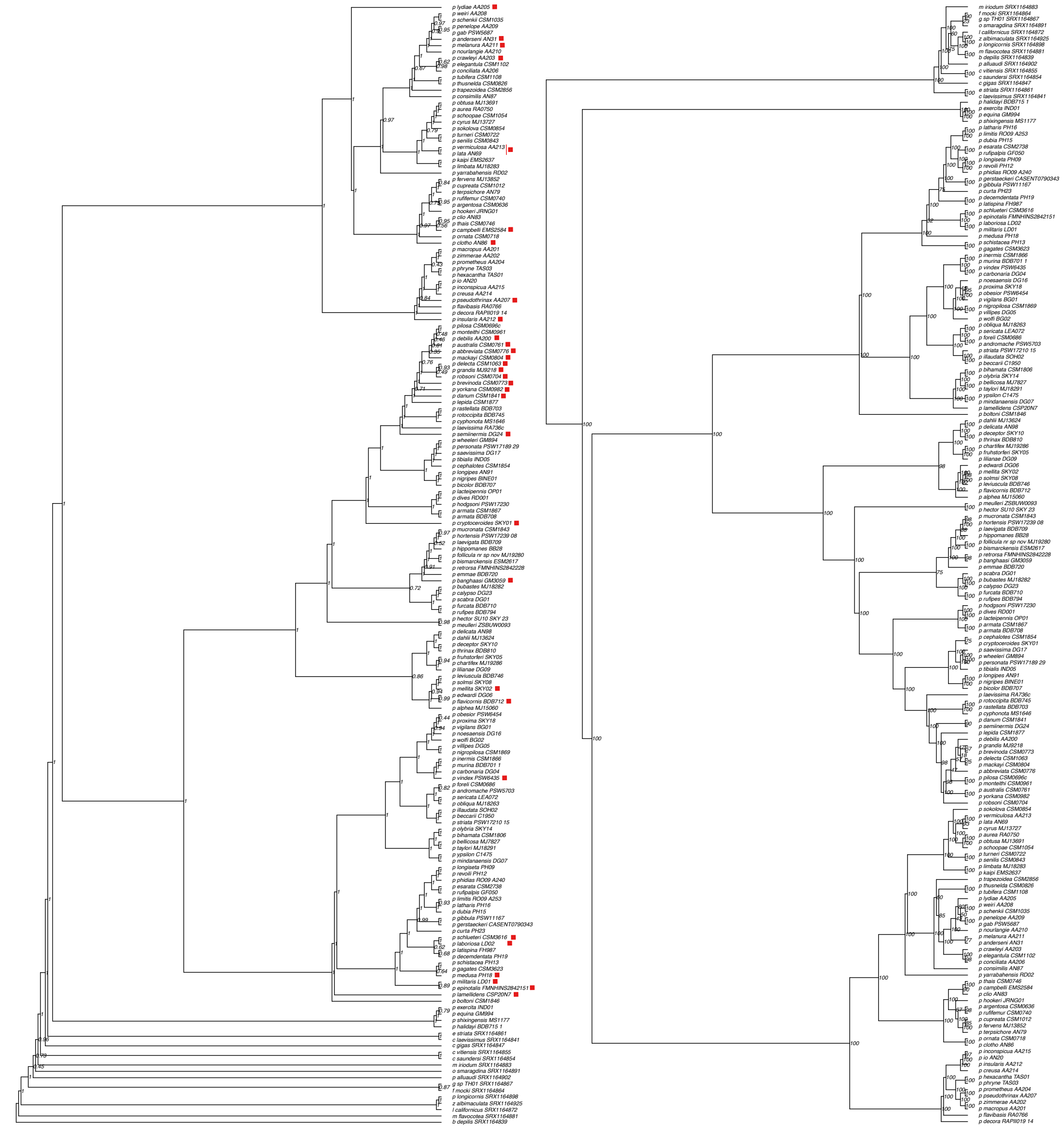


Figure 6.4: ASTRAL-III topology shown alongside the RAxML topology. Differences between the gene-tree and maximum likelihood ingroup topologies are labeled with red boxes.

Figure 6.5: Time-scaled phylogeny of Polyrhachis inferred using MCMCTree with data.

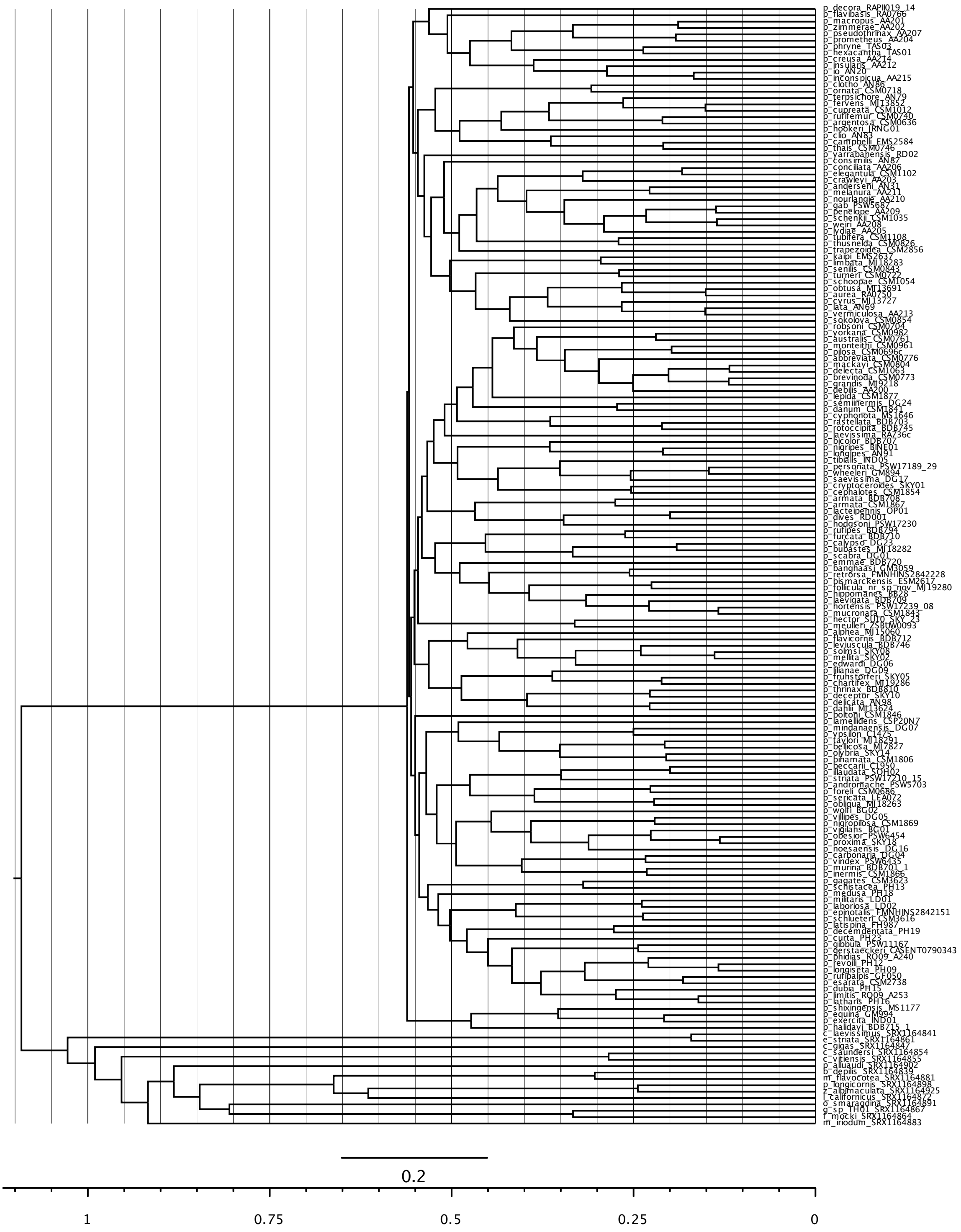
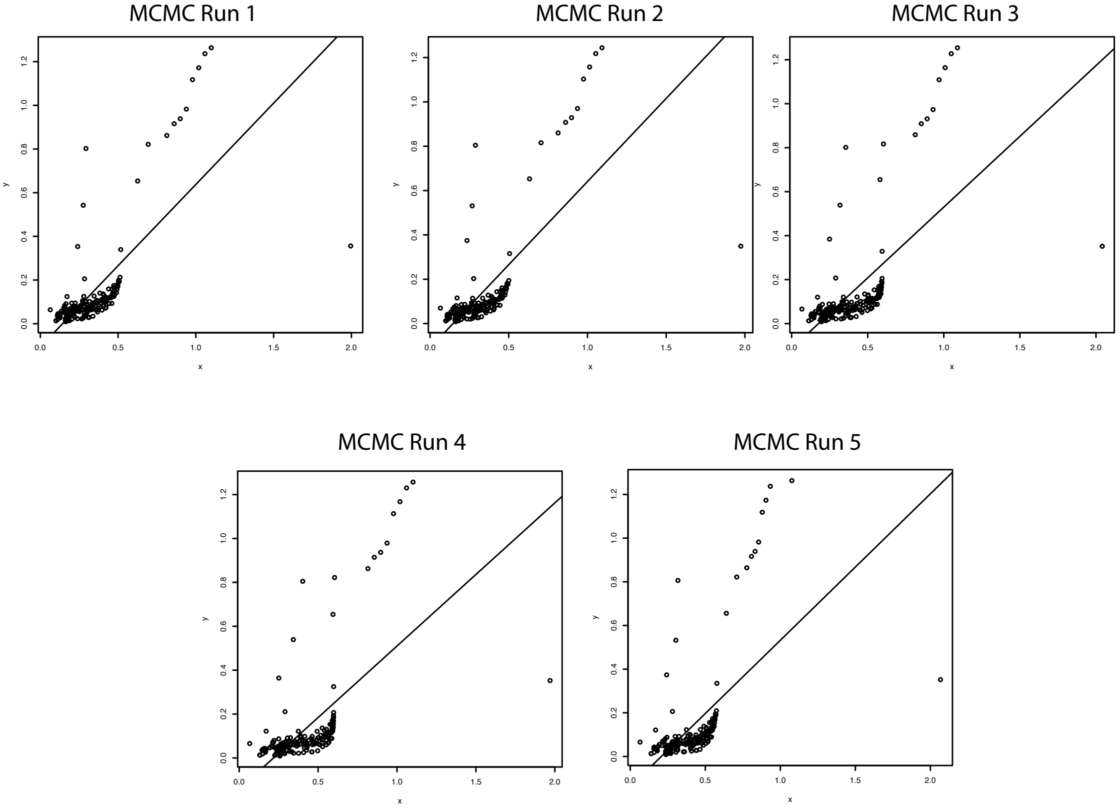


Figure 6.6: Time-scaled phylogeny of Polyrhachis inferred using MCMCTree without data (i.e. priors-only run).


MCMC Run 1

MCMC Run 2

MCMC Run 3

MCMC Run 4

MCMC Run 5

Figure 6.7: Mean 95% HPD values for with-data and no-data (i.e. priors-only) MCMC runs, plotted against each other. Note that for each independent run, values clearly deviate from the linear relationship expected if data were not informative.

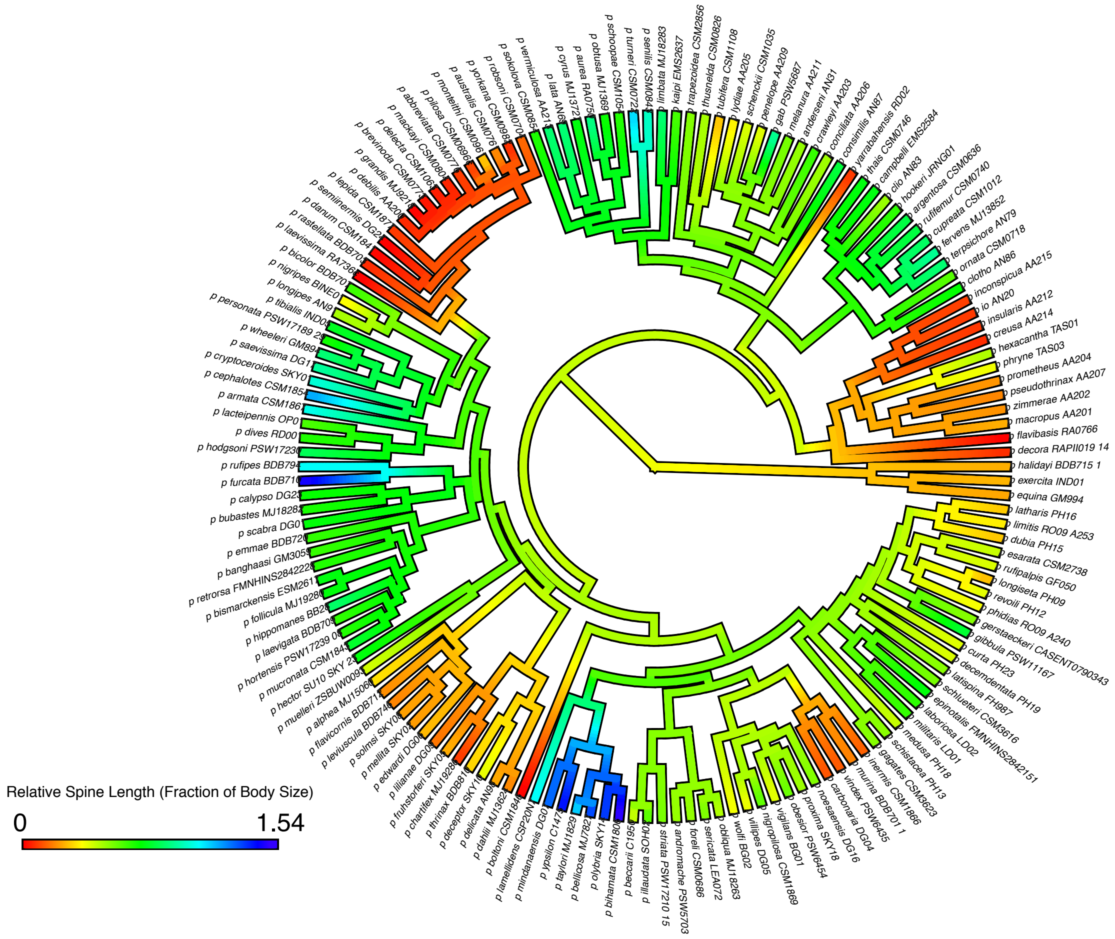


Figure 6.8: Ancestral state reconstruction for Relative Spine Length implemented using the "contMap" function in phytools.

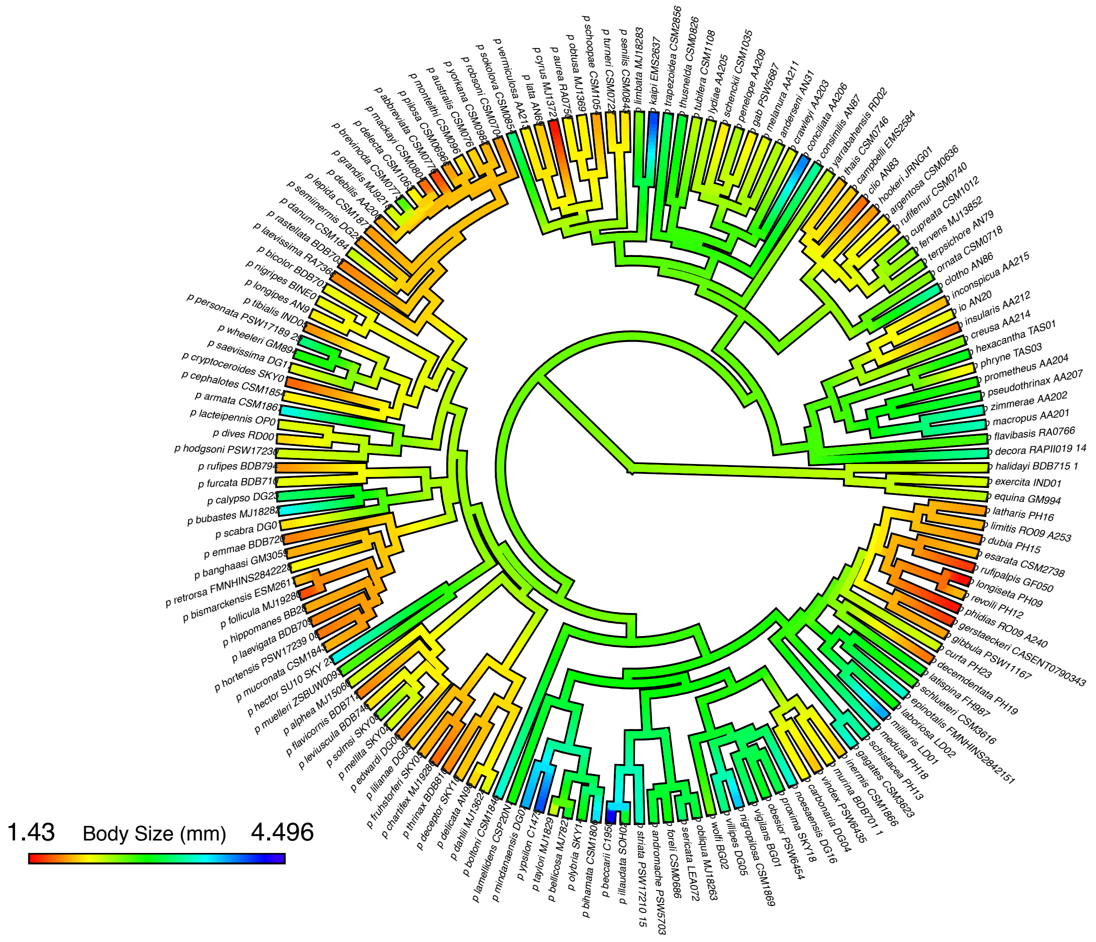


Figure 6.9: Ancestral state reconstruction for Body Size implemented using the “contMap” function in phytools.

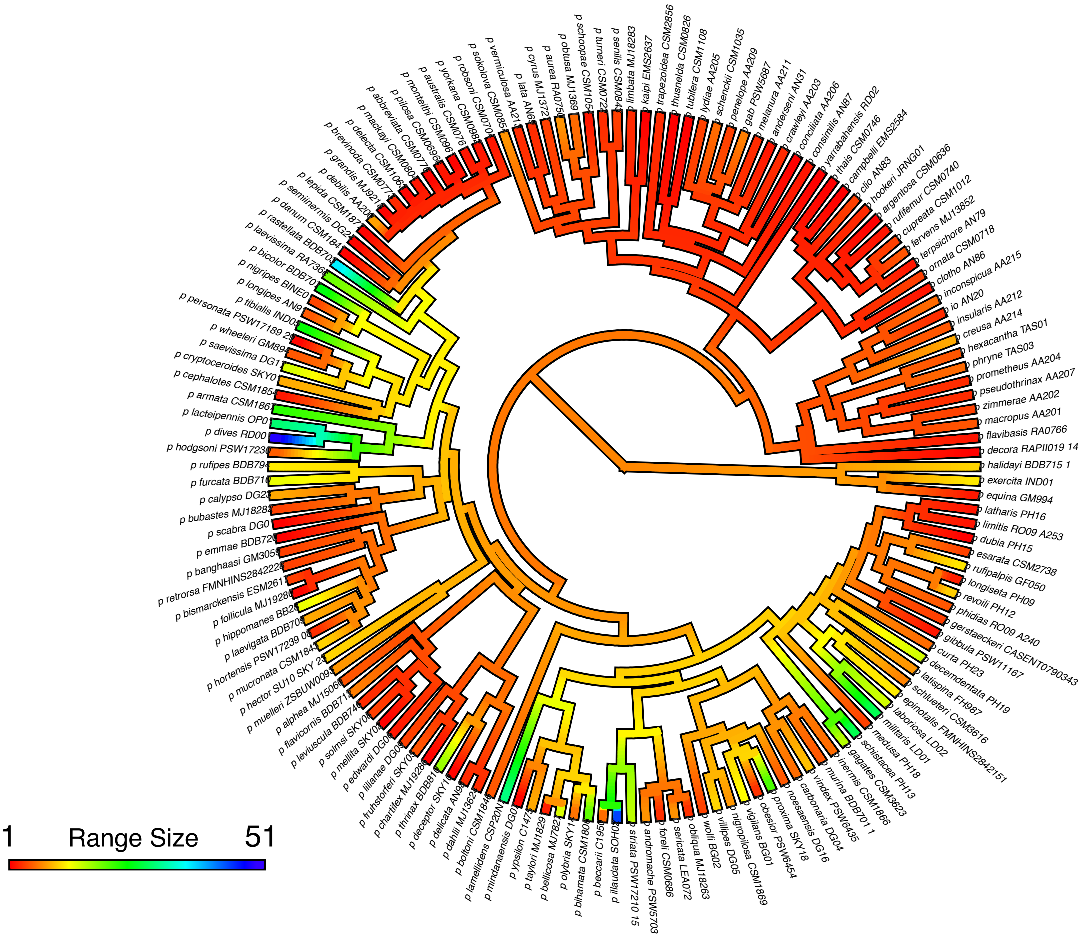


Figure 6.10: Ancestral state reconstruction for Range Size implemented using the "contMap" function in phytools.

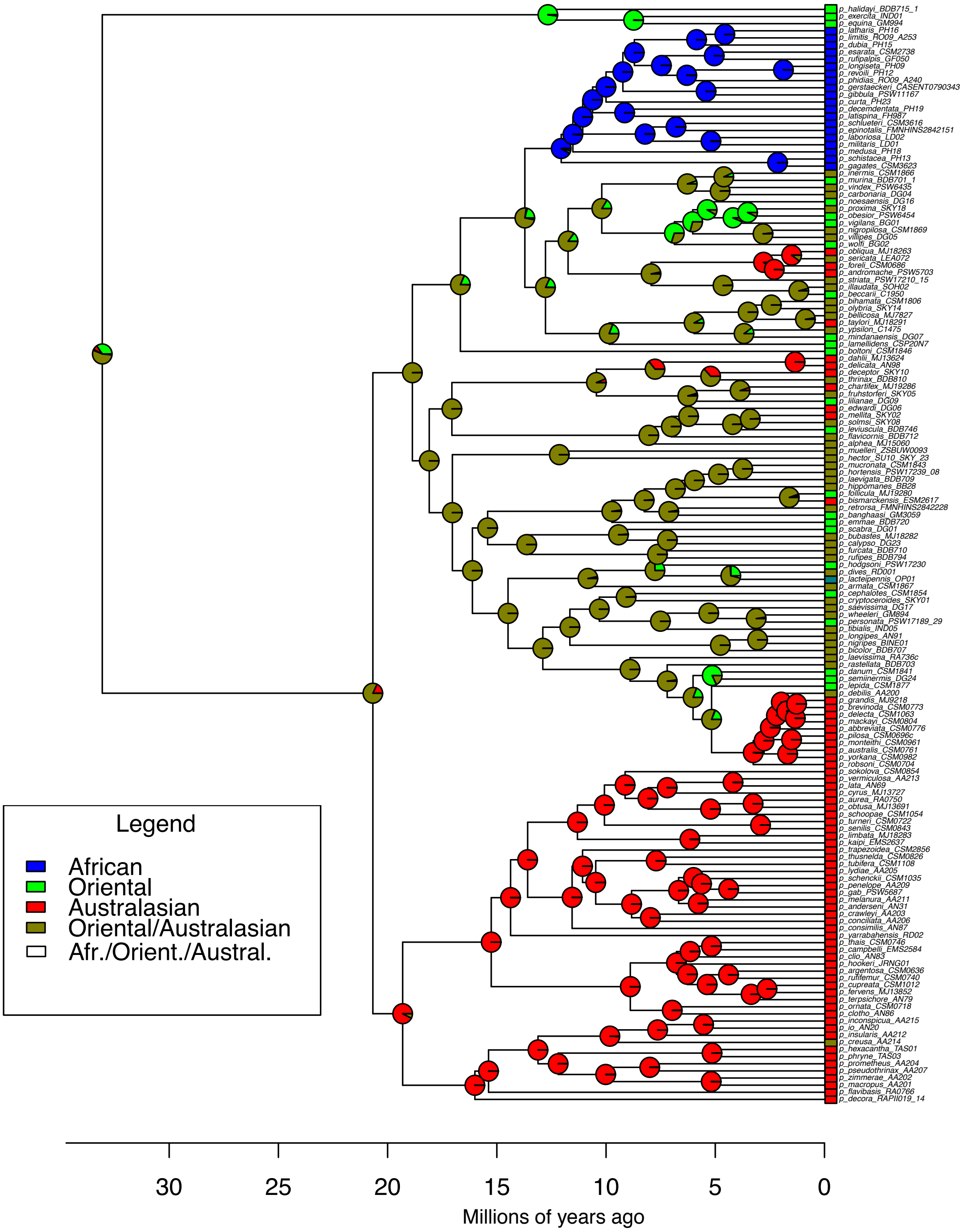


Figure 6.11: Results from ancestral range reconstruction under a DEC model implemented in BioGeoBEARS.

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