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Mutualisms drive plant trait evolution beyond interaction-related traits

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Abstract

Mutualisms have driven the evolution of extraordinary structures and behavioural traits, but their impact on traits beyond those directly involved in the interaction remains unclear. We addressed this gap using a highly evolutionarily replicated system – epiphytes in the Rubiaceae forming symbioses with ants. We employed models that allow us to test the influence of discrete mutualistic traits on continuous non-mutualistic traits. Our findings are consistent with mutualism shaping the pace of morphological evolution, strength of selection and long-term mean of non-mutualistic traits in function of mutualistic dependency. While specialised and obligate mutualisms are associated with slower trait change, less intimate, facultative and generalist mutualistic interactions – which are the most common – have a greater impact on non-mutualistic trait evolution. These results challenge the prevailing notion that mutualisms solely affect the evolution of interaction-related traits via stabilizing selection and instead demonstrate a broader role for mutualisms in shaping trait evolution.

KEYWORDS

ants, macroevolution, mutualism, phylogenetic comparative methods, plants, symbiosis, trait evolution

INTRODUCTION

Mutualisms, positive interactions that confer benefits to the species involved, are important evolutionary forces, affecting species diversification and trait evolution (Chomicki et al., 2019; Chomicki, Kiers, et al., 2020; Gómez & Verdú, 2012; Rezende et al., 2007; Sargent, 2004; Van der Niet & Johnson, 2012; Zeng & Wiens, 2021a, 2021b). Mutualisms can impact species diversification in various ways. These include partner shifts leading to divergent selection; increased partner survival; interruption of gene flow and subsequent speciation and host-symbiont incompatibility among others (Chomicki et al., 2019). Besides impacting species diversification in diverse ways, mutualisms also affect the evolution of interaction-related traits – such as showy petals involved

in the attraction of pollinators or entrance holes in plant structures provided as ant nests (domatia). Several hypotheses have been proposed to explain how this may occur.

First, a body of theoretical work posits that mutualisms should reduce the rate of phenotypic evolution in traits that are directly involved in the interaction. This should occur primarily in highly specialised and obligate mutualisms in which trait-matching phenotypes lead to strong reciprocal stabilizing selection (Kopp & Gavrilets, 2006; Raimundo et al., 2014; Thompson, 2005; Yoder & Nuismer, 2010). Empirical data support this idea. In Malpighiaceae pollinated by oil-collecting bees, the stereotyped position of floral oil glands has been maintained for tens of millions of years (Anderson, 1979; Davis et al., 2014). Likewise,

the entrance hole size in domatia provided by epiphytic Rubiaceae to symbiotic ants – which screens which symbiont can access specific domatia – is highly constrained in specialised plant species (Chomicki & Renner, 2017).

Second, another model, dating back to Darwin (1862), posits that selection may favour reciprocal increase in mutualistic trait values in interacting species. However, as Darwin was careful to point out, any seemingly matching traits might have arisen for reasons unrelated to the interaction. Studies of pollination mutualisms with extreme trait exaggeration (i.e. long nectar tubes and long-tongued pollinators) have shown that the number of pollen grains deposited increases with corolla tube length, which in theory could lead to reciprocal selection (Anderson & Johnson, 2008, 2009; Pauw et al., 2009). However, these investigations have not ruled out the possibility of sequential, unidirectional trait evolution involving multiple species and shifts in pollinators, or the influence of biotic (e.g. predation) and abiotic factors (e.g. wind or drought).

Third, changes in the partnership, such as shifts to novel partners or breakdown of the interaction, are thought to promote evolutionary change of mutualism-related traits via relaxed selection from the former partner and directional selection exerted by the new mutualistic partner (Bodbyl Roels & Kelly, 2011; Chomicki & Renner, 2017; Davis et al., 2014; Gervasi & Schiestl, 2017; Ramos & Schiestl, 2019; Whittall & Hodges, 2007). Examples come from pollination mutualisms in which populations lacking pollinators either naturally (for instance, on islands or in newly occupied environments) or experimentally, independently evolve automatic selfing – both at the micro and macroevolutionary scales (Bodbyl Roels & Kelly, 2011; Culley et al., 2002; Gervasi & Schiestl, 2017; Ramos & Schiestl, 2019).

The impact of mutualism on trait evolution – and vice versa – has focused on traits that are directly involved in the mutualism. Yet, mutualisms could affect traits beyond those involved in the interaction. This could occur through scaling relationships between traits (allometry) if for instance the benefit of a mutualistic trait – such an ant domatium developing in a plant stem – shifts the scaling relationships between leaves and stem (Brouat & McKey, 2000, 2001; Chomicki & Renner, 2019b) or drives a redistribution of nutritional resources in other, non-mutualistic traits (Zhang et al., 2011). It could also occur via functional constraints – for instance if investment in a mutualistic trait shifts trade-offs for resource allocation or defence – or via pleiotropic effects, for instance if a gene involved in the development of a mutualistic trait affects non-mutualistic traits. In addition to these insights, a modelling effort identified the so-called Red King effect, a situation where the slowest evolving partner gains the largest share of the benefits in mutualisms (Bergstrom & Lachmann, 2003). This suggests that mutualisms should drive all species traits to evolve slowly – not just interaction-related traits.

Despite these suggestions that mutualism may affect the evolution of non-mutualistic traits, this topic has received very little attention. Moreover, because specialised and obligate mutualisms appear to have the strongest stabilising effect on interaction-related traits, the degree of mutualistic dependence (Chomicki, Kiers, et al., 2020) could also influence the impact of mutualism on non-mutualistic traits. Three main limitations may have hampered progress in this area. First, inferring the evolution of mutualisms back in time relies on combinations of traits used as proxies. Some, but not all, of these traits could impact phenotypic evolution, and disentangling this is difficult. Second, the effect of mutualisms on trait evolution needs to be assessed in the context of important abiotic variables, for instance climate (Anderson & Johnson, 2008). Third, a group with sufficient evolutionary replication in its mutualistic interactions is needed to effectively study the evolution of traits.

Here, we use epiphytic ant-plants in the Hydnophytinae, a clade of ca. 105 species of Rubiaceae, to address how mutualisms influence traits not directly involved in the interaction. These plants offer nesting sites and sometimes also food rewards to ants in return for nutrients and sometimes seed dispersal and defence against herbivores (Campbell et al., 2023; Chomicki et al., 2016, 2017; Chomicki, Kadereit, et al., 2020; Chomicki & Renner, 2016a, 2016b, 2017, 2019a, 2019b). The Hydnophytinae have undergone four independent gains of highly specialised and dependent mutualisms and twelve losses of mutualisms (Chomicki et al., 2017). Knowledge of the Hydnophytinae's mutualistic trait ecology together with their high level of evolutionary replication provides an excellent opportunity to test how distinct mutualistic strategies might affect the evolution of non-mutualistic traits.

To address this question, we used an arsenal of phylogenetic evolutionary models. These models allow us to test for an association between discrete mutualistic traits and continuous non-mutualistic traits while testing for potentially confounding associations with climate variables. Specifically, we ask: (i) How does discrete mutualistic trait evolution affect the evolution of continuous non-mutualistic traits? And (ii) can the effect of mutualisms on non-mutualistic trait evolution be understood through the type of interaction (for instance, degree of partner dependence or type of symbiont) or is it trait specific? These analyses allow us to evaluate the impact of mutualisms on the evolution of traits, beyond those directly involved in the interaction, and emphasise the importance of considering mutualisms in understanding broader patterns of trait evolution.

MATERIALS AND METHODS

Taxon dataset

We expanded the taxon sampling of Chomicki and Renner (2017) and a matrix of six markers (nuclear ITS and ETS and plastid ndhF, trnH-psbA, trnL intron and trnL-trnF spacer) to include 89 of the ~105 species of Hydnophytinae. DNA extraction, amplification and sequencing procedures were performed as in Chomicki and Renner (2017). Primer sequences are provided in Table S1.

We also broadened the outgroup sampling, which now includes 46 taxa, selected to be able to include two Rubiaceae fossils that were used for time calibration (see below, section on *Molecular clock dating*). The total dataset consisted of 135 taxa (Dataset S1).

Molecular clock dating

Molecular dating analyses relied on BEAST v.1.8 (Drummond et al., 2012) and uncorrelated lognormal relaxed clock models. We used the GTR+G substitution model – determined as best model by jModelTest 2 (Darriba et al., 2012), relying on AIC – with four rate categories and a Yule tree prior, and relied on a single partition to avoid over-parametrisation. MCMCs were run for 100 million generations, with parameters and trees sampled every 10,000 generations. We used Tracer v.1.7 (Rambaut et al., 2018) to check that the effective sample size (ESS) of all parameters was >200, indicating that runs had converged. After discarding 20% as burn-in, trees were summarised in TreeAnnotator v.1.8 (part of the BEAST package) using the options 'maximum clade credibility tree', which is the tree with the highest product of the posterior probability of all its nodes, 'mean node height' and a posterior probability of 0.98. The final tree was visualised in FigTree v1.4 (Rambaut, 2012).

To calibrate the phylogeny, we constrained the age of the root to 22±7 million years (Ma), which is the age of the split between the Pacific clade and the so called *Psychotria* clade IV obtained in Barrabé et al. (2014), using a normal prior and a standard deviation of 4 corresponding to their 95% confidence interval. We used a pollen fossil of *Faramea* described by Graham (2009) and also used by Barrabé et al. (2014) to constrain the *Faramea* crown node, using a Gamma distribution with a shape prior of 2.0, a scale of 1.0 and an offset of 37.3 Ma. Thirdly, we constrained the node, including *Morinda, Mitchellia* and *Gaertnera* with a fruit fossil of *Morinda* from the Eocene found in the Changchang Basin of Hainan Island, China (Shi et al., 2012), which has been dated to the Middle Eocene (~48 to ~38 Ma) by Spicer et al. (2014). Therefore, we set the offset to 38.0 Ma and used the same Gamma shape prior and scale as above (2.0 and 1.0). Lastly, we constrained the obligate *Squamellaria* clade (*S. imberbis, S. wilsonii, S. huxleyana, S. thekii, S.*

major, S. grayi), restricted to small areas in the nearby Fijian islands Vanua Levu and Taveuni (Chomicki & Renner, 2016a) to maximally 4 Ma, the age of the older of the two islands (Sarnat, 2009). In general, geological constraints can be problematic since earlier evolution on an older island followed by extinction could explain why some taxa are older than the island in which they are endemic. However, here, the obligate *Squamellaria* clade is endemic for a narrow area in both islands, and its species have low dispersal ability due to their obligate reciprocal dependence on their specialized farming ant *Philidris nagasau* (Chomicki & Renner, 2016b). Thus, it is unlikely that the *Squamellaria* clade originated on an older island (i.e. Viti Levu) and then went extinct, and we can thus use the island emergence age as a maximum age constrain.

Trait dataset

We generated a trait dataset for the Hydnophytinae ant-plants, mostly from literature. Trait acquisition is described in detail in the Supplementary Materials (see *Trait dataset* in *Supplementary Materials and Methods*). All raw data are provided in Dataset S1, and in the GitHub repository (www.github.com/gburin/hydnoants).

Accounting for climate variables

Prior to fitting the models of trait evolution, we tested for the potential influence of similarities in climate between the sampling areas. We first used a Principal Component Analysis (PCA) using climate data from WorldClim (Fick & Hijmans, 2017). We used all 19 WorldClim variables, and after the PCA, we retained the first three Principal Components (PC1-3), as they explain more than 95% of the variance in the original data (Table S2). We tested for correlations between each continuous trait (stem area, leaf area, corolla length and petiole length) and the first three climate PCs considering the phylogenetic structure of the errors using a Phylogenetic Generalised Least Squares (PGLS) approach. Ultimately, we did not find any significant relationships after correcting for multiple tests (Tables S3–S14), so we used the variables without any transformation in all downstream analyses.

Linking rates of non-mutualistic continuous trait evolution to transitions in discrete mutualistic trait states

Joint estimation of discrete mutualistic and continuous non-mutualistic trait histories

To assess the evolutionary dynamics of the discrete mutualistic and continuous non-mutualistic traits of

interest, we used an approach that jointly estimates the likelihood for both traits (Boyko et al., 2023). This approach estimates the dynamics of both traits in combination, therefore considering possible effects of one trait on the other (Boyko et al., 2023). We considered three models of discrete trait evolution: one with equal rates between all states ('Equal Rates' – ER), one where transitions between pairs of states have the same rate but can change between pairs of states ('Symmetrical Rates' – SYM) and one in which all transition rates are different ('All Rates Different' – ARD). To model the evolution of non-mutualistic continuous traits, we employed Brownian Motion (BM) and Ornstein–Uhlenbeck (OU) models. BM quantifies random drift while OU identifies selection's impact using three parameters: strength (alpha), optimum (theta) and trait evolution rate (sigma). The Ornstein–Uhlenbeck process captures the fundamental idea that species possess distinct trait values as they adapt to distinct niches (Grabowski et al., 2023). We used six evolutionary models, all of which correspond to distinct adaptive scenarios: (i) Single-rate Brownian Motion (BM1); (ii) multiple-rate Brownian Motion (BMS); (iii) single-rate OU (OU1); (iv) OU with multiple optima but same alpha and sigma (OUM); (v) OU with multiple optima and alpha but same sigma (OUMA) and (vi) OU with multiple optima and sigma but the same alpha (OUMV).

To avoid arbitrary thresholds for selecting the best model (such as δAIC value greater than 2) for the pairwise combinations of discrete and continuous traits, after jointly fitting all combinations between discrete and continuous trait models, we used the Akaike weights of each combination to calculate averaged parameter values. We thus analysed the averaged values of each parameter to assess how different continuous traits evolved under distinct states of the discrete trait regardless of one or more 'best' models, hence avoiding any arbitrary selection criteria.

To test for differences in parameter values between different states of each discrete trait, we used phylogenetic generalised linear models. For traits with two states (namely Reward and Domatium Growth), we tested for the differences using the *pgls* function from the package *caper* (Orme et al., 2023), whereas for the traits with three states (namely Hole Diameter and Warts), we used the function *phylANOVA* from the *phytools* package (Revell, 2012). For the latter, we used simulation-based post hoc tests to identify which states had significantly different values for the parameters. The results are shown by capital letters in the bottom two rows of panels in Figures 2 and 3. All tests used the expected mean and variance values at the tips, which were estimated considering the uncertainty on the ancestral history of the discrete trait (Boyko et al., 2023).

Analyses relied on either the Maximum Clade Credibility (MCC) tree (for which we ran 100 simulations) or a set of 20 trees randomly sampled from the posterior distribution of trees (for which we ran 5 simulations for each tree, totalling 100 simulations overall – results shown in the *Supplemental Materials*). We did not use a hidden state in our analysis to maintain parameter efficiency in relation to the size of our phylogeny, which could otherwise have led to spurious results. However, the likelihood that rate heterogeneity biased our results is negligible due to the size of the phylogeny. All scripts are available at www.github.com/gburin/hydnoants.

Cross-validation of our results with OUwie

Since the approach used here is novel and has not been extensively tested, we also analysed our data using an established approach to cross-validate our results (OUwie – Beaulieu et al., 2012). For this, we used stochastic character mapping (Bollback, 2006; Huelsenbeck et al., 2003) as implemented in the R package phytools (Revell, 2012) to reconstruct evolutionary histories of each discrete trait. For the MCC tree, we assumed a model where all transition rates are empirically estimated (rather than constraining transition rates to standard models [e.g. equal rates or all rates different]) and independent of each other, which was the best model for the MCC topology. The root state was set to be sampled from the conditional scaled-likelihood distribution. For the trees sampled from the posterior distribution, we used the best model selected for each of the trees. We generated 1000 maps for the MCC tree and 100 for each of the sampled topologies from the posterior distribution and used all to summarise the states at each node to account for the many possible evolutionary histories of each trait. We analysed the proportion of each state at the main nodes to assess the ancestral state of each discrete trait.

To assess the evolutionary dynamics of the continuous traits for each of the discrete traits' state, we sampled 100 of the 1000 simulated maps in the MCC tree for each discrete mutualistic trait (and 10 for each of the sampled trees from the posterior distribution) and used them to fit the same six models of evolution for each continuous non-mutualistic trait as used above.

We again used the Akaike weight of each model to calculate averaged parameter values for each stochastic map. Lastly, we analysed both the distribution of averaged parameter values and the pairwise differences between parameters estimated within each simulation (i.e. stochastic map) and generated pseudo-posterior distributions of averaged parameter values to assess how different continuous trait states evolved under distinct states of the discrete trait. Interpretations for this set of ancillary analyses were based on visual inspection of the distributions and on the number of simulations for which the parameter value for one given state

was higher than for the other(s). Results are shown in the chord diagrams in Figures S23–S25. All scripts for the sensitivity analyses are available at www.github.com/gburin/hydnoants.

Testing the association between style length polymorphism and mutualistic strategy

Style length polymorphism is a proxy for mating type since homostyly is associated with automatic selfing (Darwin, 1897). If the presence of highly aggressive Dolichoderine ants (*Philidris* or *Anonychomyrma*) deters pollinators, then automatic selfing should be favoured in species with aggressive ants. See *Supplementary Materials* for trait data. To test whether symbiont type evolved jointly with mating type, we used Pagel's (1994) test for correlated evolution, as implemented in BayesTraits V3 (Meade & Pagel, 2017). We ran independent versus dependent models of evolution and used Bayes Factor (BF) to test for the support of the correlated model [Log Bayes Factors = 2(log marginal correlated model − log marginal likelihood uncorrelated model)].

RESULTS

Phylogenetics and molecular-clock dating of the Hydnophytinae

We first generated a new Hydnophytinae phylogeny, expanding the matrix of Chomicki and Renner (2017). Maximum likelihood and Bayesian analyses yielded a tree topology (Figure 1) congruent with Chomicki and Renner (2017) in which *Squamellaria* is sister to all remaining Hydnophytinae taxa. The next clade is the genus *Anthorrhiza*, with the newly sampled species *Hydnophytum orichalcum* placed as sister to *Anthorrhiza*, albeit with low support (but geographically consistent with *Anthorrhiza*). The molecular-clock dating analysis yielded an age for the Hydnophytinae of 18.8 ± 3 Myrs, falling with the 95% HPD of the age found by Chomicki and Renner (2017), who used a different calibration approach.

Mutualism-dependent non-mutualistic trait comparative analyses

Our analyses revealed no significant associations between the climate PCs and the continuous traits (Tables S3–S14). This indicates that climate is not a major driver of the evolution of the variation seen across our continuous traits of interest in this clade. Therefore, we ran all downstream analyses using the untransformed variables.

For most combinations between discrete and continuous traits, the best fitting models were based on OU processes (Table S15). However, due to the high number of variables and models, we opted for a model-averaging approach by calculating the weighted average of parameter values. The final value of each parameter was calculated by multiplying the estimated value for a given parameter in a given model by the Bayesian Information Criterion weight (BICw) of that model. Thus, we need not resort to any arbitrary criterion (such as δBIC >2) and can evaluate the dynamics solely based on the parameter values. We thus base our conclusions from different scenarios weighted by their respective relative support, providing a more balanced view of the underlying processes. Nevertheless, we did not include any model that had unreasonably high or low BIC values (greater than 10^4) compared to the other models for the same combination of traits since such models likely fit the data poorly and would bias the averaged parameter values.

Effect of mutualistic traits on the long-term means of non-mutualistic traits

Overall, our results show that each mutualistic traits had different levels of association with non-mutualistic traits. The type of domatium growth has the greatest impact on non-mutualistic traits. Specifically, apical domatium growth was associated with higher long-term mean values (θ) for all four non-mutualistic traits, namely corolla length, leaf area, petiole length and stem area in comparison to diffuse domatium growth (Figure 2). Similarly, the presence of food rewards (post-anthetic nectaries, Figure 1; Chomicki et al., 2016) was associated with higher long-term mean values for all four continuous non-mutualistic traits. For warts on inner domatium walls (Figure 1), species with fully differentiated warts had higher long-term means for all four continuous traits. Species that have lost warts had the lowest θ values for leaf area, petiole length and stem area but not corolla length for which species with variable warts had equally low θ value (Figure 2). For domatium entrance hole diameter (Figure 1), species with one large hole at the base had higher long-term means for leaf area, petiole length and stem area, but not corolla length. Species with all domatium holes being large had the highest long-term mean values, although differences are minute (Figure 2).

Effect of mutualistic traits on the evolutionary rates of non-mutualistic traits

The evolutionary rates (σ^2) of all continuous traits were higher in species with diffuse domatium growth compared to species with apical domatium growth
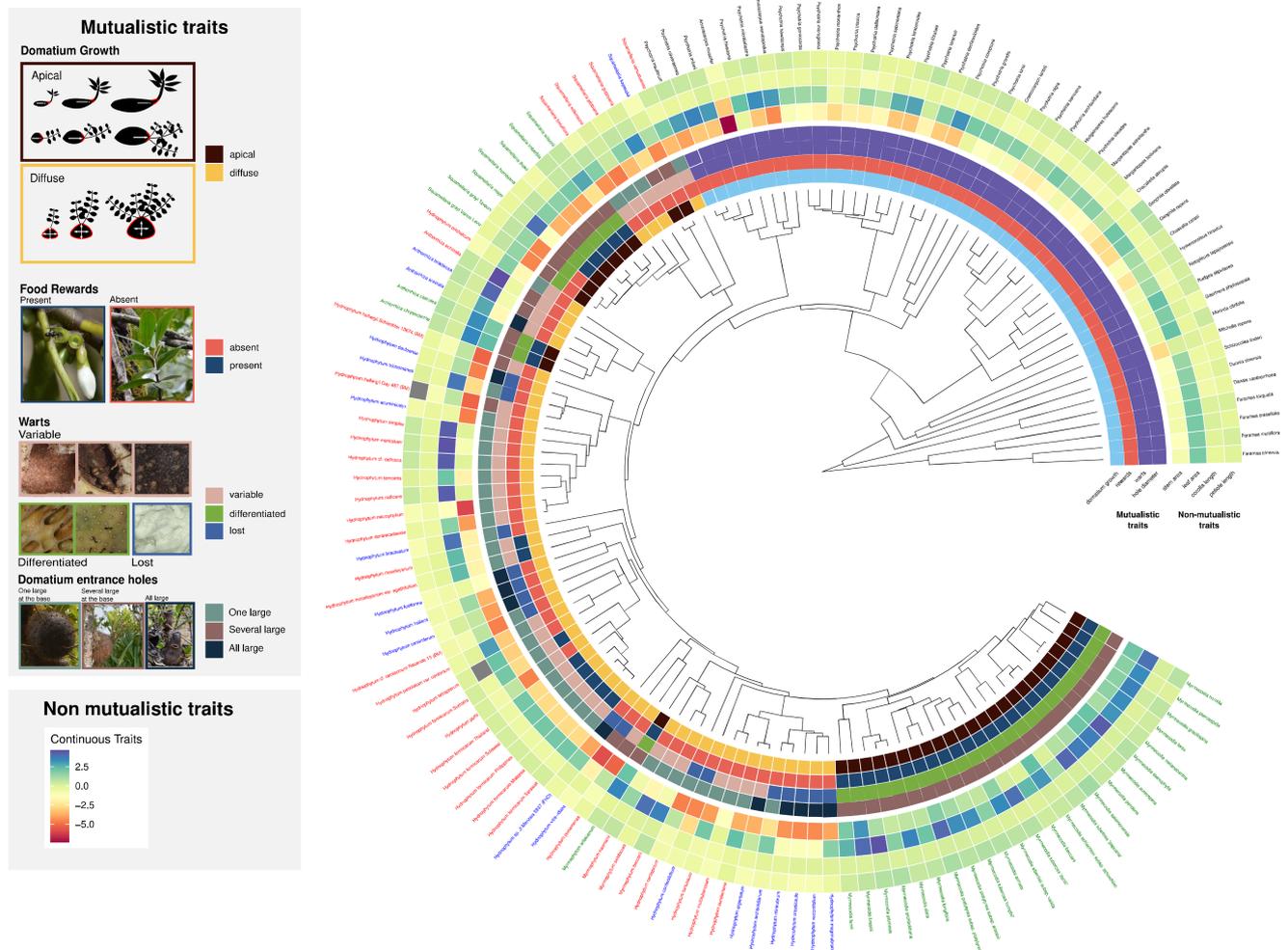
FIGURE 1 Phylogenetic distribution of the discrete mutualistic traits and continuous non-mutualistic traits analysed in this study. All traits are mapped on a dated phylogeny including ~80% of the Hydnophytinae species. Tip label colours refer to outgroups (black), facultative and generalist taxa (red), obligate and specialised taxa (green) or species having lost the symbiosis with ants (blue). The legend on the left shows the four mutualistic traits studied here. Domatium growth can be either apical, meaning that the domatium enlarges only from its apex (growing zone shown in red), and this includes taxa with a single stem (top), like most *Myrmecodia* species and others with multiple stems (bottom), like the obligate Fijian *Squamellaria* species. Food rewards, in the form of post-anthetic nectaries, can be present or absent. Inside the domatium, walls can be variable with smooth-like areas (left) interspersed with internal roots (middle) and warty-like areas (right). In other species, walls are fully differentiated with clear-cut smooth areas that are often wax covered (left) and warty areas, with evenly sized and spaced warts. Finally, entrance holes can be small with a single larger hole at the base of the domatium (left), include a mix of small and several large holes at the base, or can all be large.

(Figure 3). Similarly, species lacking food rewards had higher evolutionary rates for all traits (Figure 3). The evolutionary rate for all four non-mutualistic traits was greater in the presence of variable warts as compared to differentiated ones, and species that lost warts had the highest evolutionary rate for leaf area (Figure 3). The patterns observed for domatium entrance hole diameter were more variable, with species with a single large hole at the base having the highest evolutionary rate for corolla length, while species with several large holes at the base had the highest rates for leaf area, and stem area. For petiole length, species with either several or one large hole at the domatium base had higher rates when compared to species with all holes being large.

Effect of mutualistic traits on the strength of selection on non-mutualistic traits

The impact of mutualistic traits on the strength of selection on the four continuous non-mutualistic traits was weak (Figure 4). For all combinations of traits, there were no clear differences between the strength of selection for each state of the discrete traits. Non-mutualistic traits, such as corolla length and stem area, appear to be primarily influenced by the stochastic component of the process (i.e. Brownian Motion). This is evident in their significantly smaller estimated alpha values compared to leaf area and petiole length (Figure 4) and suggests that mutualistic interactions influence the ultimate evolutionary outcomes of these continuous traits. However,
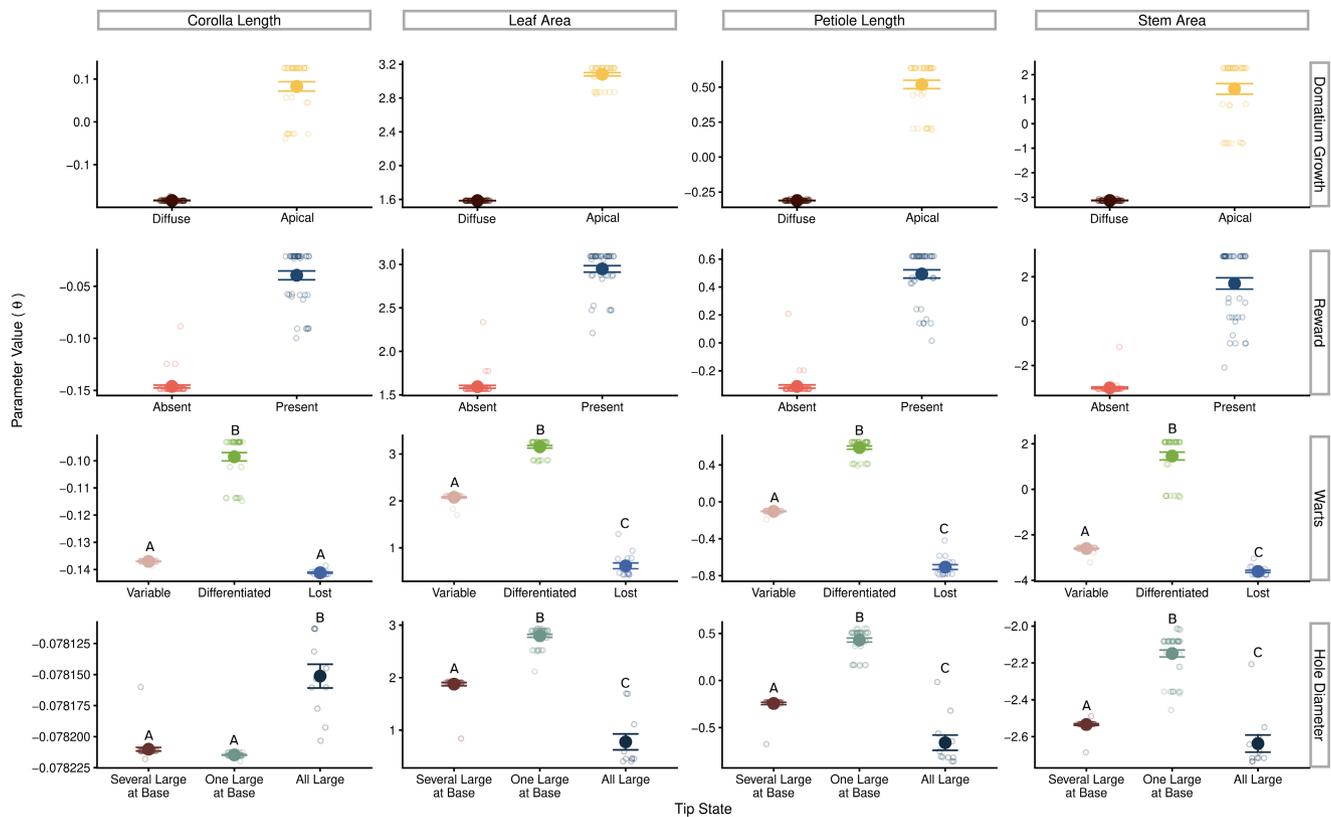
FIGURE 2 The association between discrete mutualistic traits (rows) and the long-term mean (θ) of continuous non-mutualistic traits (columns). The plots show the distribution of expected θ values for the OU-based models for each of the four continuous traits in association with different states for the four discrete mutualistic traits. All differences were statistically significant (Tables S16–S31) and the capital letters in the bottom two rows of panels indicate how the states are grouped based on the post hoc analysis of the phylogenetic ANOVA.

the speed at which these outcomes are achieved depends on the interplay between the long-term mean phenotype and the strength of selection on non-mutualistic traits.

Evaluating the impact of mutalistic strategy on floral traits

We also tested whether the type of mutualistic strategy and the presence or absence of highly aggressive Dolichoderine ants as main symbionts were correlated with style length polymorphism (as a proxy for automatic selfing vs. out-crossing; *Materials and Methods*). BayesTraits analyses revealed that the correlated model of evolution was strongly supported (Bayes Factor = 13.48), supporting the hypothesis of an association between the evolution of automatic selfing and the type of mutualistic ants (Figure S1).

Sensitivity analyses

Incorporating phylogenetic uncertainty

Analysing macroevolutionary patterns on multiple topologies can account for the uncertainty of the true evolutionary history of a group. When accounting for

phylogenetic uncertainty, we observed virtually identical patterns to the ones from the MCC tree in the main analysis when applying the same pipeline to the randomly sampled topologies (Figures S2–S17), indicating that our results are robust to phylogenetic uncertainty.

Cross-validation of our analysis using an established approach

Because the hOUwie approach is recent (Boyko et al., 2023), we also used another, more classical approach to test the association between mutualistic discrete traits and non-mutualistic, continuous traits. To do so, we followed the traditional pipeline for OUwie and first inferred the evolutionary histories of all discrete mutualistic traits using stochastic mapping (*Materials and Methods*). All ancestral state estimations are shown in Figures S18–S21. Next, we fitted the same six continuous trait evolution models to each simulation of discrete trait evolution mentioned before and inspected the results both visually and by analysing the proportion of simulations for which a given parameter value was greater or smaller than the others (represented in the chord diagrams on Figures S23–S25, but see Figure S22 for a toy example). The results from the OUwie analyses (Figures S23–S25) show the same overall
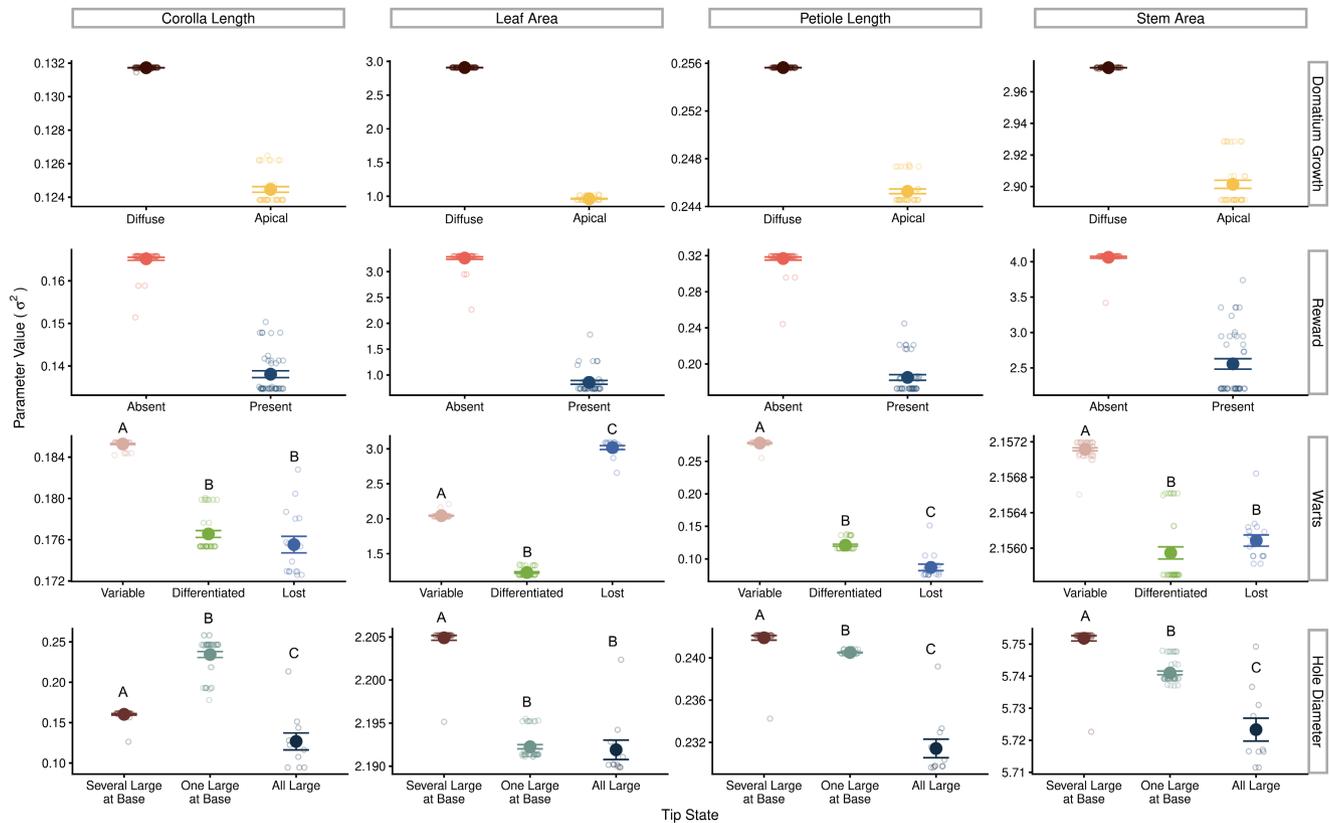
FIGURE 3 The association between discrete mutualistic traits (rows) and the evolutionary rate (σ^2) of continuous non-mutualistic traits (columns). The plots show distribution of expected σ^2 values for the OU-based models for each of the four continuous traits in association with different states for the four discrete mutualistic traits. All differences were statistically significant (Tables S32–S47) and the capital letters in the bottom two rows of panels indicate how the states are grouped based on the post hoc analysis of the phylogenetic ANOVA.

patterns observed for the main analysis, reinforcing the robustness of our results.

DISCUSSION

Our analyses reveal a consistent pattern with specialised and obligate mutualistic trait states associated with higher long-term means (θ) of non-mutualistic traits, suggesting stabilising effects of mutualisms beyond the traits directly involved in the interaction (Figures 2–4; Figures S23–S25). This suggests that highly dependent mutualisms have the potential to promote slow trait evolution, regardless of a trait's involvement in the interaction. This is in line with the Red King effect model (Bergstrom & Lachmann, 2003), which suggests that species, not simply mutualistic traits, should evolve more slowly when involved in mutualisms.

By contrast, facultative and generalist mutualisms and species that have lost the mutualism are associated with increased evolutionary rates (σ^2) in non-mutualistic traits, for instance leaf area. The acceleration of evolutionary rates in species that experienced mutualism breakdown has already been documented for traits connected to the interactions (Chomicki & Renner, 2017). Our work now suggests that this effect extends to non-mutualistic traits as well. An important

finding is that generalist and facultative mutualism are associated with high evolutionary rate values in non-mutualistic traits. This suggests that these partnerships can also drive fast evolutionary change in non-mutualistic traits, which challenges the prevailing notion that mutualisms slow trait evolution. Because facultative and generalist mutualisms are more common than highly specialised and obligate mutualisms (Batstone et al., 2018; Chomicki, Kiers, et al., 2020), this implies that mutualisms may shape trait evolution even more broadly than so far acknowledged.

Our finding that the degree of partner dependence (i.e. facultative/obligate or generalist/specialised; Chomicki, Kiers, et al., 2020) affects trait evolution differently reinforces the idea that shifts between mutualistic strategies can drive trait evolution. This has been widely documented for interaction-related traits, such as floral traits in pollination mutualisms (Bodbyl Roels & Kelly, 2011; Davis et al., 2014; Gervasi & Schiestl, 2017; Whittall & Hodges, 2007). Our work now shows that this effect goes beyond the traits that are directly involved in the interaction. Much like coevolutionary dynamics can spread to non-interacting species through indirect effects in mutualistic networks (Guimarães et al., 2017), the influence of mutualisms on trait evolution also extends to non-mutualistic traits.
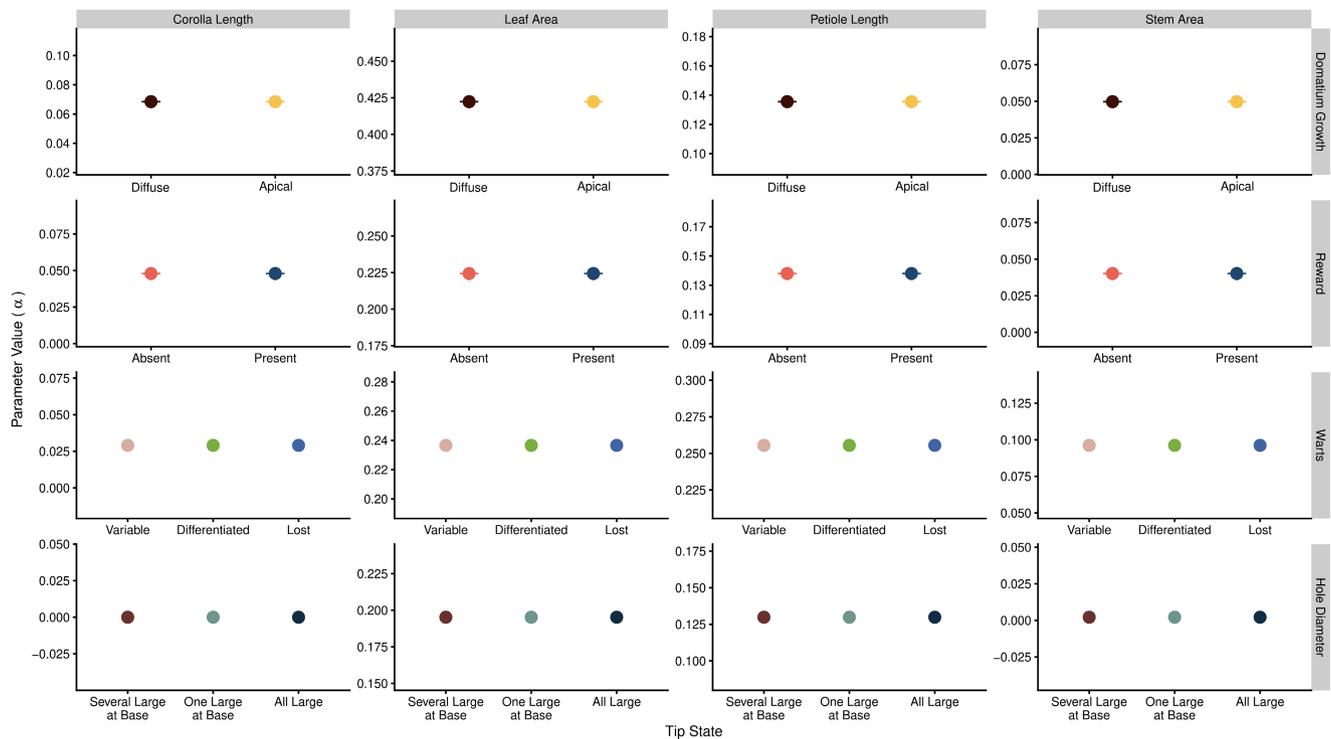
FIGURE 4 The association between discrete mutualistic traits (rows) and the strength of selection (α) on continuous non-mutualistic traits (columns). The plots show the estimated α values from the OU-based models for each of the four continuous traits in association with different states of the four discrete mutualistic traits.

It is important to recall that Ornstein–Uhlenbeck processes were initially implemented in population genetics to model features such as fitness optima (represented by theta) and stabilising selection (represented by alpha) (Lande, 1976). However, even though mathematically identical, when applied at macroevolutionary timescales, we must interpret the parameters of these models differently (Cooper et al., 2016). In deep time scales, the parameters of this type of model are representing the long-term mean phenotype and rate of adaptation towards this mean phenotype, respectively (Cooper et al., 2016; Grabowski et al., 2023). Given the different timescales, a more conservative way of interpreting the alpha parameter would be that it represents persistent directional/stabilising selection occurring at much shorter timescales towards the long-term mean phenotype.

Mutualisms can alter a species' environmental niche, for instance when a species controls the dispersal of another (Chomicki, Kadereit, et al., 2020). Our findings suggest that mutualisms can also affect the partners' environmental niches through their impact on the evolution of non-mutualistic traits. In our case, the evolution of leaf area was strongly affected by mutualistic traits (Figures 2 and 3). Because leaf area is under environmental constraints (Wright et al., 2017), this suggests that mutualism may alter the outcome of environmental sorting. Similarly, because mutualistic interactions are mediated by species traits, the evolutionary effects on

non-mutualistic traits might generate eco-evolutionary feedbacks, thus affecting the mutualistic interactions directly.

Our analyses show that mutualistic traits can affect the evolutionary dynamics of non-mutualistic traits. This raises the question of how this occurs. One possibility is that mutualistic traits that are costly – such as a long corolla restricting nectar access or a domatium with entrance holes restricting partner entry – change a species' allometric balance. This may influence the ability of other traits to change, making subsequent reversions difficult. Studies of leaf/stem allometry in clades forming mutualisms with ants (Brouat & McKey, 2000, 2001; Chomicki & Renner, 2019b), support this idea. Chomicki and Renner (2019b) identified repeated leaf/stem allometry shifts in specialised lineages of the Hydnophytinae, congruent with our analyses. Another way is through pleiotropy. In many mutualisms, resource exchange entails pleiotropic costs. For instance, in the legume-rhizobia symbiosis, mutualistic rhizobia invest energy in nitrogen fixation, enhancing host fitness but sacrificing energy storage compounds, such as polyhydroxybutyrate (PHB) and rhizopines, which are important for offspring survival (Kiers & Denison, 2008; Oono et al., 2011). Similarly, functional and developmental constraints and trade-offs can also limit the potential evolutionary changes in traits by imposing inherent limitations on their adaptability and thereby influencing the dynamics of trait evolution.

Another potential link between traits directly involved in mutualisms and non-mutualistic traits could come from the behaviour of animal mutualists. Aggressive symbiotic ants, for instance, may influence which pollinators can be attracted, and this has led to the evolution of avoidance strategies to repel ants from inflorescences in *Acacia* (Gaume et al., 2005; Ghazoul, 2001; Raine et al., 2002). Here, we found that homostyly, a trait often associated with automatic selfing (Darwin, 1897), predominates in ant-plants that form symbioses with aggressive Dolichoderine ants (Figure S1), matching expectations that aggressive symbionts can scare away pollinators.

CONCLUSION

This study supports that mutualisms impact trait evolution beyond the traits directly involved in the interaction, likely through allometric relationships (in our case, leaf and stem sizes) and effects mediated by partner behaviour (aggressive symbionts and floral traits). Our findings also match a key prediction of the Red King theory proposed by Bergstrom and Lachmann (2003), which posits that species engaged in mutualistic relationships should generally evolve slowly. That the effects of mutualistic traits on non-mutualistic traits are strongest in obligate and specialised mutualisms are consistent with stabilising selection of non-mutualistic traits, in line with other microevolutionary and simulation studies (Kopp & Gavrilets, 2006; Raimundo et al., 2014; Thompson, 2005; Yoder & Nuismer, 2010). On the other hand, facultative and generalist mutualisms appear to favour faster trait evolution. Future research should explore the underlying connections among traits to uncover functional, molecular, and physiological mechanisms behind the observed patterns.

AUTHOR CONTRIBUTIONS
G.B. and G.C. conceived the study; G.B., L.C.E.C. and G.C. conducted analyses; G.C. and S.S.R. acquired funding for sequencing; G.C., G.B., L.C.E.C., S.S.R. E.T.K. wrote the manuscript.

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DATA AVAILABILITY STATEMENT
DNA sequences are available on NCBI, and the data and code that support the findings of this study are openly available in Zenodo at https://zenodo.org/doi/10.5281/zenodo.10405028.

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