

The relationship between chlorophyllous spores and mycorrhizal associations in ferns: evidence from an evolutionary approach

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Abstract

Premise: Approximately 14% of all fern species have physiologically active chlorophyllous spores that are much more short-lived than the more common and dormant achlorophyllous spores. Most chlorophyllous-spored species (70%) are epiphytes and account for almost 37% of all epiphytic ferns. Chlorophyllous-spored ferns are also overrepresented among fern species in habitats with waterlogged soils, of which nearly 60% have chlorophyllous spores. Ferns in these disparate habitat types also have a low incidence of mycorrhizal associations. We therefore hypothesized that autotrophic chlorophyllous spores represent an adaptation of ferns to habitats with scarce mycorrhizal associations.

Methods: We evaluated the coevolution of chlorophyllous spores and mycorrhizal associations in ferns and their relation to habitat type using phylogenetic comparative methods.

Results: Although we did not find support for the coevolution of spore type and mycorrhizal associations, we did find that chlorophyllous spores and the absence of mycorrhizal associations have coevolved with epiphytic and waterlogged habitats. Transition rates to epiphytic and waterlogged habitats were significantly higher in species with chlorophyllous spores compared to achlorophyllous lineages.

Conclusions: Spore type and mycorrhizal associations appear to play important roles in the radiation of ferns into different habitat types. Future work should focus on clarifying the functional significance of these associations.

KEYWORDS

arbuscular mycorrhizae, chlorophyllous spores, correlated evolution, dark septate endophytes, epiphytes, grammitid, green spores, pteridophytes

Mature fern spores can be either chlorophyllous or achlorophyllous; that is, they may or may not contain functioning chloroplasts. Although achlorophyllous spores (hereafter AS) predominate, chlorophyllous spores (hereafter CS) occur in ca. 14% (~1500 species) of extant fern species and are known to occur in all species of the

families Equisetaceae, Hymenophyllaceae, Onocleaceae, Osmundaceae, the grammitid group in Polypodiaceae, in some species of other polypod genera (*Aglaomorpha*, *Loxogramme*, *Platyserium*, *Pleopeltis*, *Pleurosoriopsis*), and in some species of *Elaphoglossum* (Dryopteridaceae), *Polytaenium* (Pteridaceae), *Lomaria* (Blechnaceae), and

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Lomariopsis (Lomariopsidaceae) (Lloyd and Klekowski, 1970; Sundue et al., 2011; Mellado-Mansilla et al., 2021). Associated with the presence of chloroplasts are a lack of dormancy, short viability (on average, 48 days), and rapid germination (Lloyd and Klekowski, 1970). In contrast, AS can remain viable in the soil spore bank for years (Lloyd and Klekowski, 1970). Accordingly, CS have long been considered to have a low tolerance to stressful environmental conditions (Lloyd and Klekowski, 1970; Ballesteros et al., 2017). Recent studies, however, have demonstrated that CS can tolerate water stress as well as their counterparts do (Ballesteros et al., 2017; López-Pozo et al., 2019a). Complex mechanisms to avoid photo-damage have also been reported, at least in terrestrial fern taxa (López-Pozo et al., 2019b). Nevertheless, it remains unclear why ferns have evolved CS, considering that their short period of viability could limit both dispersal and successful population establishment. Why then, have CS evolved multiple times across the fern phylogeny, including in some of the most species-rich clades?

Some features of the biology of fern lineages with a high incidence of CS provide potential insight into this apparent paradox. First, most ferns with CS belong to two ecologically distinct functional groups. Members of one group are epiphytes, particularly abundant in humid tropical forests at middle to high elevations (Lloyd and Klekowski, 1970; Sundue et al., 2011); CS are present in all or almost all members of some of the most species-rich epiphytic fern lineages, including the grammitid ferns (Polypodiaceae), the filmy ferns (Hymenophyllaceae), and some clades of *Lomariopsis* (Lomariopsidaceae) and *Elaphoglossum* (Dryopteridaceae) (Sundue et al., 2011). The other group of predominately CS species are ferns such as in Onocleaceae, Osmundaceae, and Equisetaceae that occur in waterlogged soils, particularly in temperate regions. Although these relationships have been noted by previous authors (Lloyd and Klekowski, 1970; Hill and Wagner, 1974), they remain unexamined in an evolutionary or ecological context.

Second, despite the limited viability of CS, fern species with this spore type are overrepresented on oceanic islands, especially among species of the family Hymenophyllaceae and the grammitids (Dassler and Farrar, 2001). Although Dassler and Farrar (2001) did not consider the evolutionary history of the species with CS inhabiting those islands (i.e., colonization, diversification, and extinction events), Sundue et al. (2014) suggested that Hawaiian grammitid flora could be the result of multiple long-distance migrations. This pattern has long puzzled fern biogeographers, since the short life span of CS, along with their assumed susceptibility to low temperatures and UV radiation, suggests that they are poorly suited for long-distance dispersal (Tryon, 1970, 1986).

Based on these observations, it has been hypothesized that the lack of dormancy of CS could be a key trait to conquer novel habitats such as the forest canopy or islands by allowing them to germinate shortly after being released (Lloyd and Klekowski, 1970; Sundue et al., 2015), whereas AS often take weeks or months to germinate (Lloyd and Klekowski, 1970).

The success of chlorophyllous-spored species of Hymenophyllaceae and grammitids in colonizing new habitats has also been linked to the morphology and ecology of their gametophytes, which are chlorophyllous, noncordiform, long-lived, and have asexual dispersal by gemmae (Stokey and Atkinson, 1958; Atkinson, 1960; Yoroi, 1971; Dassler and Farrar, 2001; Pinson et al., 2017). However, similar gametophytes can also be found in epiphytic taxa with AS such as in vittarioid ferns (Pteridaceae) (Dassler and Farrar, 2001; Nitta et al., 2020). From an evolutionary standpoint, the ability to access canopy or island habitats might offer new ecological opportunity, thus fuelling rapid diversification of lineages with CS according to the expectations of adaptive radiation theory (Simpson, 1953; Bouchenak-Khelladi et al., 2015). Thus, the presence of CS might represent an important precursor trait in highly diverse epiphytic lineages.

In the present study, we propose a novel hypothesis to explain the evolution of CS, namely that mycorrhizal associations play an important and overlooked role in the evolution and distribution of CS in ferns. Although mycorrhizae play a crucial role for plants in enhancing nutrient and water uptake (Willis et al., 2013), about one third of all fern species lack mycorrhizal associations (Lehnert et al., 2017), which is a much higher proportion than in angiosperms (~15%) and gymnosperms (0%) (Wang and Qiu, 2006). It has been hypothesized that ferns have a lower dependency on mycorrhizal associations because their growth is more limited by carbon than by nutrient availability (Kessler et al., 2014). Additionally, it has been observed that nonmycorrhizal fern species are overrepresented among epiphytes and species from waterlogged soils (Lehnert et al., 2017; Brundrett and Tedersoo, 2018), the same groups that frequently possess CS. The paucity of mycorrhiza-fern associations appears to be driven by the fact that mycorrhizae are uncommon in the dynamic forest canopies that lack well-developed soils (Lehnert et al., 2017; Brundrett and Tedersoo, 2018), whereas in waterlogged soils fungi cannot grow under anoxic conditions (Helgason and Fitter, 2009; Tedersoo, 2017). Evidence also suggests that spore dispersal of arbuscular mycorrhizal fungi is limited, and therefore, they may be poor colonizers of epiphytic habitats (Willis et al., 2013).

A possible link between chlorophyll in spores and mycorrhizal associations is further indicated by the observation that mycorrhizal dependency acts as a filter in the colonization of oceanic islands by angiosperms (Delavaux et al., 2019): Since specialized mycorrhizal fungi cannot establish without a host plant and a specialized host plant cannot persist without mycorrhizae, the arrival of plants from a continental flora to islands favors those species that are independent of mycorrhizal associations. Thus, we hypothesize that the successful colonization of ferns with CS on oceanic islands might be linked to possible independence from mycorrhizal fungi to germinate.

In this regard, the autotrophic condition of CS marks independence from the need for a fungal partner for spore germination and early gametophyte development. Although very little is known about the relationships of fern

gametophytes and mycorrhizal fungi, such an association appears to be common at this stage of the life cycle in many ferns (Turnau et al., 2005; Reyes Jaramillo et al., 2008; Muthukumar and Prabha, 2012; Ogura-Tsujita et al., 2013, 2019) or may even be required in species with subterranean achlorophyllous, mycoheterotrophic gametophytes, which obtain both nutrients and carbohydrates from their fungal partners (Cox et al., 2003; Whittier, 2006; Bonfante and Genre, 2008). Although fungal relationships have also been described in aboveground gametophytes (Campbell, 1908; Turnau et al., 2005; Martinez et al., 2012; Muthukumar and Prabha, 2012; Ogura-Tsujita et al., 2013; Pressel et al., 2016), these associations do not appear to be as strict as those observed for subterranean gametophytes. However, none of these studies have so far taken into account the spore type, so that the association of spore type (AS vs. CS) and gametophyte–mycorrhiza associations have not been examined directly. Interestingly, noncordate fern gametophytes, which are more common in species with CS (but see Ogura-Tsujita et al., 2013 for *Osmunda* as an exception), are characterized by the absence of fungal associations (Pressel et al., 2016). Since in ferns the presence of CS and noncordate gametophytes seem to be correlated, we suspect that the fungal-free condition reported for this type of gametophyte could also be attributable to their CS.

Based on these considerations, we hypothesized that CS represent an adaptation to habitats where the establishment

of mycorrhizal associations with gametophytes are unlikely because spores with chloroplasts can establish and grow independently of mycorrhizal fungi. To assess this general hypothesis, in the present study, we tested two sets of specific hypotheses (Table 1). In the first set, we evaluated previous findings on richness differences of species with CS in different habitats and the associations of these species with mycorrhizal fungi. Specifically, in the first set, we tested whether (H1) fern species with CS are overrepresented in epiphytic habitats and in waterlogged soils and whether (H2) fern species with CS have significantly fewer associations with mycorrhizal fungi than ferns with AS. In the second set of hypotheses, we evaluated the evolutionary relationships among species with CS, their habitats, and their mycorrhizal associations. Specifically, we tested whether (H3) spore type has evolved along with the presence or absence of mycorrhizal associations in fern lineages. Assuming that the spore type and the presence or absence of a mycorrhizal association influence the habitat inhabited by ferns, we tested whether (H4) the spore type and the mycorrhizal associations coevolved with the habitats. Finally, assuming that CS provide an evolutionary advantage in habitats with few mycorrhizae, we hypothesized that (H5) evolutionary transitions to epiphytic and waterlogged habitats are more frequent in lineages with CS and that (H6) CS increase the speciation rates in ferns, especially those living as epiphytes or in waterlogged habitats.

TABLE 1 Hypotheses and methods used to test the evolutionary relationships of ferns with chlorophyllous spores, mycorrhizal associations, and habitats.

Hypothesis	Prediction/Method	Support
(H1) Fern species with CS are overrepresented in epiphytic habitats and in waterlogged soils.	<ul style="list-style-type: none"> Differences in the number of species with CS in each habitat tested by χ^2 test Relationships explored by phylogenetic logistic regressions 	Supported. Tables 2 and 6
(H2) Fern species with CS have significantly fewer associations with mycorrhizal fungi than ferns with AS do.	<ul style="list-style-type: none"> Differences in the number of species with CS with and without mycorrhiza versus species with AS tested by χ^2 test Relationships explored by phylogenetic logistic regressions 	Supported. Tables 3 and 7
(H3) Spore type evolved along with the presence or absence of mycorrhizal associations.	<ul style="list-style-type: none"> Correlated evolution analyses for the spore type and the presence or absence of mycorrhizal associations Relationships explored by phylogenetic logistic regressions 	Not supported. Tables 4 and 8
(H4) Spore type and the mycorrhizal associations coevolved with the habitats	<ul style="list-style-type: none"> Correlated evolution analyses for the spore type/mycorrhizal associations and the habitats used by ferns Relationships explored by phylogenetic logistic regressions 	Partly supported: correlated evolution of spore type and habitats supported for epiphytic lineages and for waterlogged soils plus epiphytic habitats (habitats poor in mycorrhizae), but not for waterlogged habitats on their own. Tables 4, 5, 8, and 9
(H5) Evolutionary transitions to epiphytic and waterlogged habitats are more frequent in lineages with CS.	Transition rates estimated from correlated evolution analyses and multistate speciation and extinction models	Supported. Figures 3 and 4
(H6) CS increase the speciation rates in ferns, especially those living as epiphytes or in waterlogged habitats.	Multistate speciation and extinction models with hidden states (MuHISSES)	Not supported. Table 10

MATERIALS AND METHODS

Phylogeny and trait data

We used the most complete phylogeny on ferns currently available from Hernández-Rojas et al. (2021). This time-calibrated phylogenetic tree includes 5075 fern species and is based on six chloroplast markers (*atpB*, *rbcL*, *rps4*, *rps4-trnS* IGS, *trnL*, *trnL-trnF* IGS), 26 fossils, and three phylogenetic methods to resolve phylogenetic relationships (maximum likelihood, penalized likelihood, and relaxed-clock model) (Testo and Sundue, 2016; Hernández-Rojas et al., 2021). Information on spore type was obtained from a systematic bibliographic review for all extant species of the genera belonging to the class Polypodiopsida (Mellado-Mansilla et al., 2021). The list considered studies in which the authors described the spores from direct observations or provided photographs. The data set accounted for 1864 species (37%) present in the phylogeny. Altogether, the list includes 370 (20%) species with CS and 1494 (80%) species with AS.

We scored the habitat (epiphytic, terrestrial, waterlogged soils) of the species in the phylogenetic tree using literature references (e.g., Sundue et al., 2015; Zotz et al., 2021) and personal field experience. We scored as epiphytes all those fern species which structurally depend on other plants throughout all stages of their life cycle (Zotz, 2016). Although we based our information on epiphytism according to Zotz et al. (2021), we restricted our final list to all those fern species growing exclusively as epiphytes in their complete distributional range and also excluded facultative epiphytes (Appendix S1). We used “terrestrial” to refer only to terrestrial taxa that occur principally outside of waterlogged soils; we also included saxicolous species in this group. The term “waterlogged” was used to group all those fern species preferentially growing in wetlands, swamps, seeps, or seasonally flooded habitats. We excluded climbers (78 species), hemiepiphytes (75 species) (Zotz et al., 2021) (see Appendix S1 for details), and aquatic species (Salviniales, 82 species) due to their ecological and evolutionary differences from the other lifestyles studied here, since our main interest was epiphytism and because the low number of species in these categories limits statistical inference. Because we were interested in epiphytic, terrestrial, and waterlogged habitats, but the spore type was a binary trait (CS or AS), we rearranged these three habitats in four binary groups: (a) epiphytes (1) and terrestrials plus species from waterlogged habitats (0), (b) epiphytes plus species from waterlogged habitats (1) and terrestrials (0), (c) species from waterlogged habitats (1) and terrestrials (0), and (d) epiphytes (1) and terrestrials (0). Habitat “b” grouped epiphytes and species from waterlogged habitats in one category as a proxy for those fern species inhabiting habitats poor in mycorrhizal associations (hereafter we will refer to this habitat group as “habitats poor in mycorrhizae”). Group “c” excluded epiphytic species, and group “d” excluded species from waterlogged habitats.

Information on mycorrhizal associations (presence/absence) was obtained from Lehnert et al. (2017). The data set compiled information on observations of sporophytes and considered as “presence” all types of mycorrhization including arbuscular mycorrhizal fungi, dark septate endophytes, and mixed colonizations such as septate and aseptate endophytes. These records accounted for 710 species in our phylogenetic tree. We used the function `match.phylo.data` from the R package `Picante` v. 1.8.2 (Kembel et al., 2010) to prune the phylogeny of species lacking complete trait data; the resulting phylogeny was used for all subsequent analyses. All analyses were performed in R version 4.1.1 (R Core Team, 2021).

Statistical analyses

To test our first set of hypotheses on the differences in species richness per each trait, we used χ^2 tests, which allowed us to also include species not present in the phylogeny, thus resulting in a higher sample size. For our first hypothesis on differences in the number of fern species with CS by habitat type (H1), we used a 2×2 table containing the number of the species in each of four categories per each habitat group (e.g., number of epiphytic species with CS, number of epiphytic species with AS, number of terrestrial species with CS, and number of terrestrial species with AS). We repeated this same design to test whether the numbers of species with CS and of those lacking mycorrhizal associations were significantly different from the number of fern species with AS and mycorrhizal associations (H2). To implement these tests, we used the function `chisq.test` in the R package `stats` v. 3.6.3 (R Core Team, 2021). We also assessed the relationships among character states incorporating the phylogeny; see the section on phylogenetic logistic regressions below.

Phylogenetic comparative methods

For the second set of hypotheses, we applied phylogenetic comparative methods. To test whether the traits evolved together or independently following our hypotheses H3 and H4, we scored species with CS as 1 and species with AS with 0, and the habitats in the four combinations outlined above. We then tested for correlated evolution of the spore type against the four groups of habitats using `BayesTraits` v. 3.0.2 (Meade and Pagel, 2019). We used the functions `Discrete Dependent` and `Discrete Independent` and the `Markov chain Monte Carlo (MCMC)` method with 1,010,000 iterations and discarded the first 10,000 as burn-in. The functions simulated models in which, respectively, two traits evolved together (`Discrete Dependent`) or independently (`Discrete Independent`) (Pagel, 1994). We compared the log marginal likelihoods of the two models using a likelihood ratio test and obtained the Bayes factor value. Given a Bayes factor value >2 , the complex model (correlated evolution) should be favored (Meade and Pagel, 2019). We repeated this

analysis to test whether the mycorrhizal associations have a correlated evolution with the four different groups of habitats, and with the spore type.

To obtain complementary information on trait relationships (H1, H2, H3, H4), we tested whether spore type and mycorrhizal associations were associated with habitat type by performing phylogenetic logistic regressions with the function `phyloglm` of the R package `phylolm` v. 2.6.2 (Tung et al., 2014). We tested single-predictor models using the method `logistic_MPLE`, with 100 bootstrap replicates, and the different habitat groups as a response variable. We used the same function to test whether the presence or absence of mycorrhizal associations and the spore type were associated with the habitats but used multipredictor models with the different habitat groups as the response variable. We used the Akaike information criterion to select the best-fitted model (Akaike, 1974) and the function `r2.lik` from the R package `rr2` v. 1.0.2 (Ives and Li, 2018; Ives, 2019) to obtain the R^2 values of the models.

To test whether evolutionary transitions to epiphytic and waterlogged habitats are more frequent in lineages with CS (H5), we compared the transition rates obtained from the correlated evolution analyses (discrete dependent models favored by Bayes Factor) to those from a multistate speciation and extinction (MuSSE) model implemented in the R package `DIVERSITREE` v. 09.15 (FitzJohn, 2012). We used MuSSE models exclusively to account for the effect of the speciation and extinction process of the fern phylogeny on the transition rates of our coevolution models. For this, we established four character states to score species based on the combination of the two binary traits (spore type and habitat) used in the other analyses. We obtained exponential priors from the highest transition rate from a maximum likelihood analysis for each model using the `find.mle` function (Depaoli et al., 2020). Then, we ran four independent MCMC chains for 10,000 generations and discarded the first 1000 generations of each chain as burn-in. We used the R package `CODA` v. 0.19-4 (Plummer et al., 2006) to plot and check the convergence of each MCMC run.

Finally, to evaluate the effect of chlorophyllous spores and habitat on diversification rates of ferns (H6) we used MuHISSE (multistate speciation and extinction with hidden states). MuHISSE allows for “hidden states” to influence the diversification rates, thus offering a more realistic null hypothesis compared to equal diversification across clades (Caetano et al., 2018; Nakov et al., 2019). We fitted a total of seven models using the same four states of our traits used in the MuSSE models described above. In the “dull null” model, the turnover and extinction fraction were the same for all the four observed states. We also fitted a MuSSE model where the turnover rates were unlinked to our states. We ran two MuHISSE models which included one hidden trait (two hidden states). One model was a dependent MuHISSE constricting the diversification to be dependent on our states, and the other one was an independent MuHISSE model where the turnover was unlinked to our

states. To contrast our complex models and avoid false positives, we also fitted three multicharacter independent diversification models (MuCIDs) with increasing numbers of hidden states (4, 6, and 8), which assume that diversification rates are associated only with hidden states. We selected the best model according to the best AIC value. We ran all these models using the R package `hisse` v. 2.1.6 (Beaulieu and O'Meara, 2016).

The proportion of sampled species (`sampling.f`) by each character was calculated considering all the extant species belonging to the class Polypodiopsida excluding the aquatic families Salvinaceae and Marsileaceae, following species counts provided by Hassler (2019). Although we lack a comprehensive list of the fern species with CS, we estimated their number to be 1478 species based on the results of Mellado-Mansilla et al. (2021) (Appendix S1). Due to the uncertainty of the estimation of extinction rates using only extant species (Rabosky, 2010; Louca and Pennell, 2020; Pagel, 2020; Joly and Schoen, 2021), we constrained the extinction rates to be the same in all states. Double-step changes (i.e., transition from being an epiphyte with CS through a terrestrial species with AS) were not allowed following Pagel (1994).

RESULTS

Representation of ferns with CS in different habitats

Our final data set included 1758 fern species with phylogenetic, spore type, and habitat information (Figures 1 and 2). Of 361 species with CS, 296 species (82%) were epiphytes, 46 species (13%) were terrestrial, and 19 species (5%) inhabited waterlogged soils (Figure 2A). Of the 1397 species with AS, 433 (31%) were epiphytes, 951 (68%) were terrestrial, and 13 species (1%) lived in waterlogged soils (Figure 2A). The species richness per each character state was significantly different from each other (χ^2 test, $p < 0.05$, Table 2). The proportions of the number of species per character state of the traits used in this study and their differences were similar to those calculated for all extant fern species (Figure 2B, Table 2).

Relationship of species with CS and mycorrhizal associations per habitats

Information on mycorrhizal associations was recorded for 708 species (Figure 2C). Of the 422 fern species with mycorrhizal associations, 61 (14%) species were epiphytes, 348 (83%) were terrestrial, and 13 (3%) occurred in waterlogged habitats. In contrast, nonmycorrhizal fern species were overrepresented in the epiphytic habitat with 169 (59%) species, compared to 107 (38%) species in terrestrial and 10 (3%) species in waterlogged habitats.

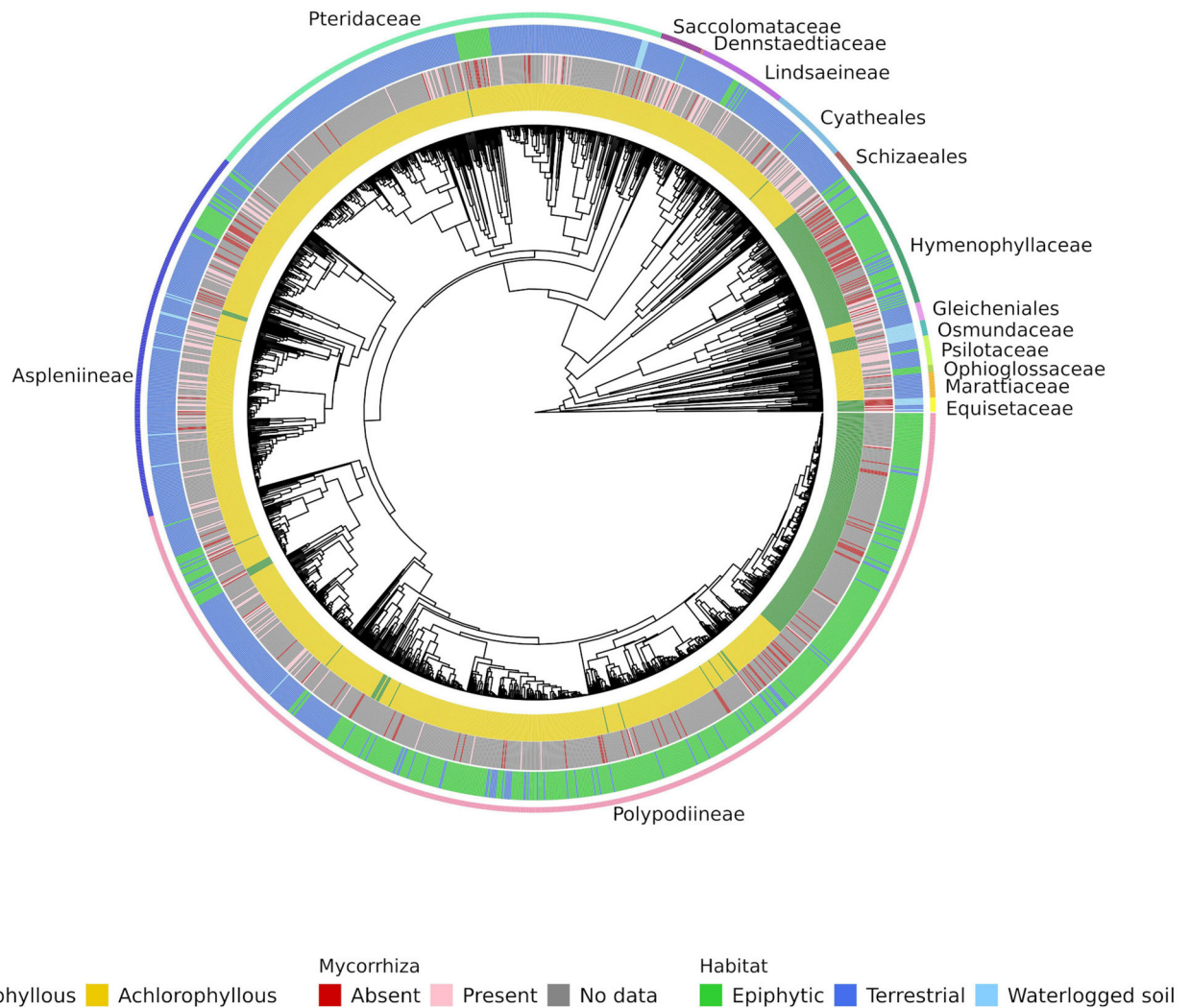


FIGURE 1 Phylogenetic tree with 1758 fern species. From inner circle to outer circle: (1) spore type, (2) mycorrhizal associations, and (3) habitat.

Concerning the proportions of species with mycorrhizal associations and their spore type, information on both traits was available for 419 species in our data set (Figures 1 and 2D), corresponding to 27 (6%) CS species with mycorrhizal associations, 80 (19%) species with CS but without mycorrhizal associations, 199 (47%) species with AS and with mycorrhizal associations, and 113 (27%) species with AS but without mycorrhizal associations. Except for the comparison of mycorrhizal associations with waterlogged habitats, all these proportions were significantly different (χ^2 tests, $p < 0.05$, Table 3).

Correlated evolution of traits

We did not find statistical evidence for correlated evolution of mycorrhizal associations and spore type (Bayes factor = -7; Table 4). However, almost all dependent models of correlated evolution of mycorrhizal associations and habitats were strongly supported by the log-marginal likelihood ratio tests

(Bayes factor >80), showing that the presence or absence of mycorrhizal associations depends on the habitat and vice versa, supporting an evolutionary correlation of these traits. The only exception (Bayes factor = -10) occurred when we evaluated the correlated evolution of mycorrhizal associations and habitat "c" (species exclusively inhabiting waterlogged habitats vs. terrestrial species) (Table 4). The dependent models of correlated evolution between spore type and habitats poor in mycorrhizae (habitat "b"), and between spore type and species growing exclusively as epiphytes (epiphytes vs. terrestrial, excluding species from waterlogged habitats, group habitat "d") were favored according to the log-marginal likelihood ratio test in BayesTraits (Table 5). This result indicated that a shift in spore type was associated with a shift in the habitat used and vice versa. We did not find support for correlated evolution of spore type among taxa growing exclusively in waterlogged habitats (waterlogged vs. terrestrial, habitat "c") nor when we considered species from waterlogged habitats within the terrestrial habitat (habitat "a") (Table 5).

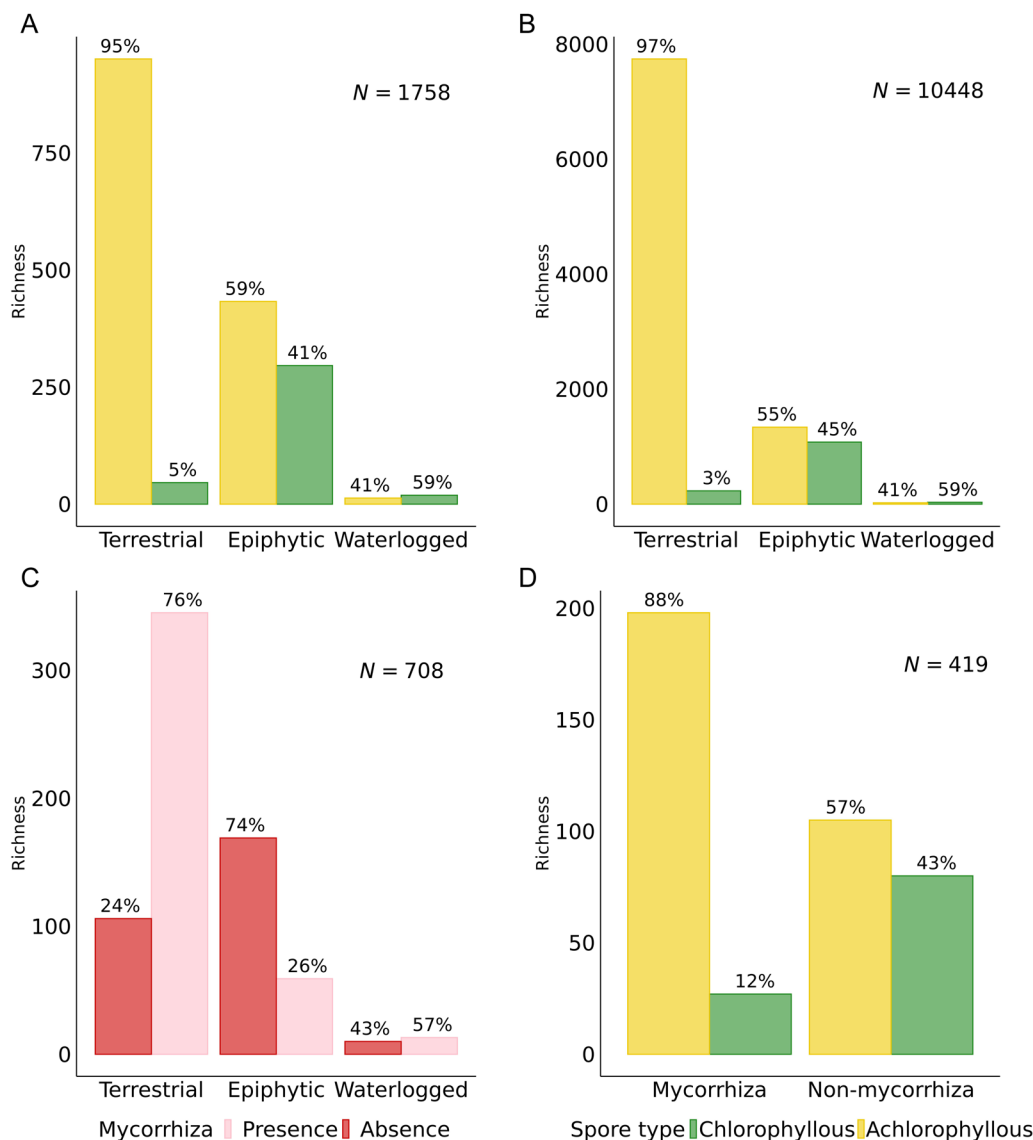


FIGURE 2 (A) Number of species in the phylogeny with and without chlorophyllous spores per habitat. (B) Estimated number of extant species of ferns with and without chlorophyllous spores per habitat, where total number does not account for Salviniales. (C) Number of species in the phylogenetic tree with data on mycorrhizal associations per habitat. (D) Number of species in the phylogenetic tree with and without chlorophyllous spores with and without mycorrhizal associations. *N* indicates the total number of species.

Complementary phylogenetic logistic regressions

Phylogenetic logistic regression models of spore type and habitats showed a strong effect of spore type on the habitat of the fern species ($P < 0.05$). The sole exception was found when testing the influence of spore type in lineages exclusively comparing species from waterlogged habitats vs. terrestrial species (habitat “c”) (Table 6), in accordance with the results of the BayesTraits analyses. Our logistic regression on spore type and mycorrhizal presence or absence was significant ($P < 0.05$) but explained only around 33% of the data variance (Table 6). Our single-predictor model to test the relationships of mycorrhizal associations and habitats showed a strong effect between these two traits

($P < 0.05$), with the sole exception when testing waterlogged species separately ($P = 0.33$) (Table 7).

We also used phylogenetic logistic regressions to test the influence of mycorrhizal associations and spore type on the two groups of habitats supported by BayesTraits and logistic regressions (epiphytic vs. terrestrial excluding species from waterlogged habitats, and habitats poor in mycorrhizae vs. terrestrial) (Appendix S1). These analyses showed that mycorrhizal associations had a negative effect ($P \leq 0.05$) on the epiphytic habitat and on terrestrial habitats hampering the development of mycorrhizal fungi. These results suggested that the presence of mycorrhizae was negatively associated with lineages inhabiting these habitats. On the other hand, CS had a positive effect on the same habitat groups ($P \leq 0.05$) (Tables 8 and 9).

TABLE 2 χ^2 tests for the number of species with chlorophyllous and achlorophyllous spores in each one of the habitat groups. The number of species corresponds to values shown in Figure 2A and B. Each test is based on a 2 × 2 table.

Traits	df	χ^2	<i>p</i>
Species in the phylogeny (<i>N</i> = 1758)			
Spore type (chlorophyllous/achlorophyllous) vs.			
Epiphytic/Terrestrial + Waterlogged	1	305.3	<0.001
Epiphytic + Waterlogged/Terrestrial	1	355.5	<0.001
Waterlogged/Terrestrial	1	157.1	<0.001
Epiphytic/Terrestrial	1	341.0	<0.001
All extant fern species (<i>N</i> = 10,448)			
Spore type (chlorophyllous/achlorophyllous) vs.			
Epiphytic/Terrestrial + Waterlogged	1	2831	<0.001
Epiphytic + Waterlogged/Terrestrial	1	2976.8	<0.001
Waterlogged/Terrestrial	1	531.6	<0.001
Epiphytic/Terrestrial	1	2928.5	<0.001

TABLE 3 χ^2 tests for the number of species with mycorrhizal associations and without mycorrhizal associations in each category of spore type and in each one of the habitats groups. The number of species tested corresponds to values shown in Figure 2C.

Traits	df	χ^2	<i>p</i>
Mycorrhizal associations (presence/absence) vs.			
Chlorophyllous/Achlorophyllous	1	46.1	<0.001
Epiphytic/Terrestrial + Waterlogged	1	146.9	<0.001
Epiphytic + Waterlogged/Terrestrial	1	148.7	<0.001
Waterlogged/Terrestrial	1	3.7	0.05
Epiphytic/Terrestrial	1	156.5	<0.001

TABLE 4 Correlated evolution between mycorrhizal associations and spore types, and mycorrhizal associations and different habitat groups. LML_dep: log marginal likelihood of dependent model, LML_ind: log marginal likelihood of independent model. LBF: LogBayes factor.

Traits	LML_dep	LML_ind	LBF	Interpretation
Mycorrhizae (presence: 1)/(absence: 0) vs.				
Chlorophyllous (1)/Achlorophyllous (0)	-328.9	-325.5	-7	Weak evidence
Epiphytic(1)/Terrestrial + Waterlogged (0)	-654.3	-701.3	94	Very strong evidence
Epiphytic + Waterlogged (1)/Terrestrial (0)	-701.9	-744.5	85	Very strong evidence
Waterlogged (1)/Terrestrial (0)	-358.3	-353.1	-10	Weak evidence
Epiphytic (1)/Terrestrial (0)	-633.7	-688.6	110	Very strong evidence

Evolutionary transition rates and diversification

The parameter estimates of the correlated evolution from BayesTraits analyses showed that species with CS living terrestrially had significantly higher transition rates (15.8 events per million years) toward habitats poor in mycorrhizae than species with AS (0.001 events per million years; posterior probability = 0.98) (Figure 3A). This pattern did not change when compared to our MuSSE models accounting for speciation and extinction processes: both models indicated that the transition rates toward habitats poor in mycorrhizae were higher in lineages with CS than in those with AS (0.0006 events per million years, posterior probability = 0.99) (Figure 4A). These results did not change notably when comparing only epiphytic vs. terrestrial species (Figures 3B and 4B). On the other hand, the SSE models including hidden states showed that MuCID models with eight hidden states were favored with AIC weights of 0.99 (Table 10), suggesting that other traits not evaluated in this study might have influenced diversification rates in fern evolution.

DISCUSSION

Our results provide strong evidence that species with CS are overrepresented in epiphytic and waterlogged habitats and that ferns with CS have fewer mycorrhizal associations than species with AS. However, we did not find evidence supporting that CS and mycorrhizal associations evolved together. Instead, we found an indirect relationship in that mycorrhizal associations and the different habitats used by ferns evolved together, as suggested in previous studies (Lehnert et al., 2017; Brundrett and Tedersoo, 2018), and that CS evolved with habitats poor in mycorrhizae (Figures 3 and 4).

As in any study of phylogenetic comparative methods, methodological limitations must be considered (Davis et al., 2013; Rabosky and Goldberg, 2015; Pagel, 2020). To deal with these limitations, we used different approaches: tests of correlated evolution, phylogenetic logistic regressions, and SSE models analyses, the results of which largely

TABLE 5 Correlated evolution analyses of spore type (chlorophyllous, achlorophyllous) in fern spores and different habitat groups. LML_dep: Log marginal likelihood of dependent model, LML_ind: Log marginal likelihood of independent model. LBF: LogBayes Factor.

Traits	LML_dep	LML_ind	LBF	Interpretation
Chlorophyllous (1)/Achlorophyllous (0) vs.				
Epiphytic (1)/Terrestrial + Waterlogged (0)	-695.8	-675.6	-40	Weak evidence
Epiphytic + Waterlogged (1)/Terrestrial (0)	-706.4	-727.3	42	Very strong evidence
Waterlogged (1)/Terrestrial (0)	-189.8	-164.4	-51	Weak evidence
Epiphytic (1)/Terrestrial (0)	-662.7	-669.9	14	Very strong evidence

TABLE 6 Complementary analyses of associations between spore type and groups of habitats using phylogenetic logistic regressions. Habitat.a: epiphyte (1)/terrestrial + waterlogged (0), Habitat.b: epiphyte + waterlogged (1)/terrestrial (0), Habitat.c: waterlogged (1)/terrestrial (0), Habitat.d: epiphyte (1)/terrestrial (0). Spore type: Chlorophyllous/Achlorophyllous. Mycorrhiza: Presence (1), Absence (0).

Model	Parameters	Estimate	95% CI Lower, Upper	z	P
Habitat.a ~ SporeType	(Intercept)	-1.16	-1.35, 0	-1.49	0.1
R ² = 0.77	Chlorophyllous	0.85	0, 0.96	2.31	<0.05
Habitat.b ~ SporeType	(Intercept)	-0.04	-0.43, 0.28	-0.05	0.95
R ² = 0.76	Chlorophyllous	1.98	0, 2.66	5.14	<0.0001
Habitat.c ~ SporeType	(Intercept)	-0.19	-4.01, 1.05	-0.12	0.9
R ² = 0.53	Chlorophyllous	0.10	-0.03, 1.38	0.41	0.7
Habitat.d ~ SporeType	(Intercept)	-0.17	-0.67, 0.13	-0.17	0.9
R ² = 0.78	Chlorophyllous	1.94	0, 2.67	4.71	<0.0001
Mycorrhiza ~ SporeType	(Intercept)	0.69	0.39, 1.11	2.81	0.005
R ² = 0.33	Chlorophyllous	-1.12	-1.58, -0.56	-3.21	<0.05

TABLE 7 Complementary analyses of associations between mycorrhizal associations and groups of habitats using phylogenetic logistic regressions. Habitat.a: epiphyte (1)/terrestrial + waterlogged (0), Habitat.b: epiphyte + waterlogged (1)/terrestrial (0), Habitat.c: waterlogged (1)/terrestrial (0), Habitat.d: epiphyte (1)/terrestrial (0). Mycorrhiza: Presence (1), Absence (0).

Model	Parameters	Estimate	95% CI Lower, Upper	z	P
Habitat.a ~ Mycorrhiza	(Intercept)	-1.3	-1.95-0.46	-2.05	0.04
R ² = 0.69	Mycorrhiza1	-0.67	-0.99-0.33	-2.73	<0.05
Habitat.b ~ Mycorrhiza	(Intercept)	-0.59	-1.06, 0.09	-1.3	0.95
R ² = 0.62	Mycorrhiza1	-0.66	-0.88, -0.43	-3.92	<0.0001
Habitat.c ~ Mycorrhiza	(Intercept)	0.03	-2.77, 1.47	0.02	0.98
R ² = 0.31	Mycorrhiza1	0.08	-0.05, 0.29	0.97	0.33
Habitat.d ~ Mycorrhiza	(Intercept)	-1.19	2, -0.35	-1.93	0.05
R ² = 0.69	Mycorrhiza1	-0.69	0, 2.67	-2.89	<0.05

supported each other. Nevertheless, we found some contradictory results in the analysis of correlated evolution. Despite the significant results of the χ^2 tests and logistic regressions testing the relationship between spore type and mycorrhizal associations, we found no support for their correlated evolution. The same occurred when considering spore type and species from waterlogged habitats alone or within the terrestrial habitat, although small species

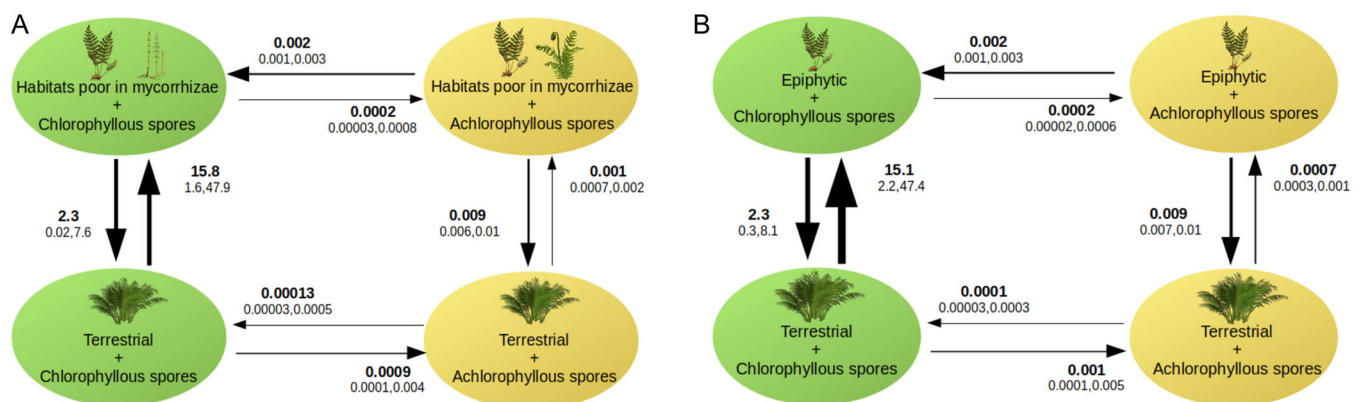
numbers (21 CS, 12 AS) limit the statistical power of these analyses. It is important to note that these phylogenetic methods can establish a correlation between traits but not causality and that evolution is a complex process involving not one but a suite of traits and processes. Considering these caveats, we will carefully discuss the implication of CS in the establishment and evolution of ferns inhabiting habitats poor in mycorrhizae.

TABLE 8 Model with interaction between spore type and mycorrhizal association in habitats poor in mycorrhizae. Habitat b: Epiphytes + waterlogged (1)/terrestrial (0).

Habitat_b ~ SporeType * Mycorrhizal $R^2 = 0.64$	Estimate	95% CI Lower, Upper	z	P
(Intercept)	-0.04	-0.68, 0.60	-0.05	1
Chlorophyllous	2.49	1.39, 4.25	3.74	0.0002
Mycorrhiza_presence	-0.72	-1.27, -0.36	-2.48	0.01
Chlorophyllous:Mycorrhiza_presence	-0.75	-2.0, 0.65	-1.10	0.3

TABLE 9 Model with interaction between spore type and mycorrhizal association. Habitat d: epiphytes (1)/terrestrial (0).

Habitat_d ~ SporeType * Mycorrhizal $R^2 = 0.71$	Estimate	95% CI Lower, Upper	z	P
(Intercept)	-0.75	-1.66, -0.10	-0.96	0.3
Chlorophyllous	1.26	0.64, 2.10	2.24	0.03
Mycorrhiza_presence	-0.51	-1.13, -0.03	-2.06	0.04
Chlorophyllous:Mycorrhiza_presence	-0.90	-2.12, 0.16	-1.36	0.2

**FIGURE 3** Estimation of transition rates (per million years) for correlated evolution of spore type and habit. Medians were estimated from the posterior distribution. The ranges in parentheses represent the 95% highest posterior density. (A) Model of correlated evolution between habitats poor in mycorrhiza and spore type. (B) Model of correlated evolution between epiphytic and terrestrial habitats, and spore type. All values were obtained from BayesTraits v. 3.0.2 (Meade and Pagel, 2019). Images from [pngwing.com](https://www.pngwing.com).

First, our results supported our hypothesis H1 on the overrepresentation of CS in epiphytic and waterlogged habitats. Most of the species with CS in our phylogeny were epiphytes (82%), but this high percentage is also impressive when considering the total extant diversity of ferns with CS: around 70% of them (~1080 species) were epiphytes, accounting for at least 45% of global epiphytic fern species richness scored in this study and would represent ~37% of the richness of epiphytic ferns considered by Zotz et al. (2021). Among terrestrial species most of the species with CS (~87%) belonged to the families Hymenophyllaceae and Polypodiaceae; most of these taxa grow on rocks rather than soil itself. Conversely, most of the terrestrial chlorophyllous-spored species belonging to Osmundaceae, Equisetaceae,

and Onocleaceae grow in periodically or constantly waterlogged soils (Figures 1 and 2A) in the temperate zones (Sundue et al., 2011). These species represent ~56% of the total richness inhabiting such habitats (Figures 1 and 2).

We also found support for our hypothesis H2 that species with CS have significantly fewer mycorrhizal associations, because a higher proportion of ferns with CS were free of mycorrhizae (75% of the total CS species sampled) when compared to AS species (36%). Although this finding could support our main hypothesis that CS represents an adaptation of ferns to habitats where mycorrhizae are scarce, we did not find strong evidence for hypothesis H3 on the correlated evolution between spore type and mycorrhizal associations despite the

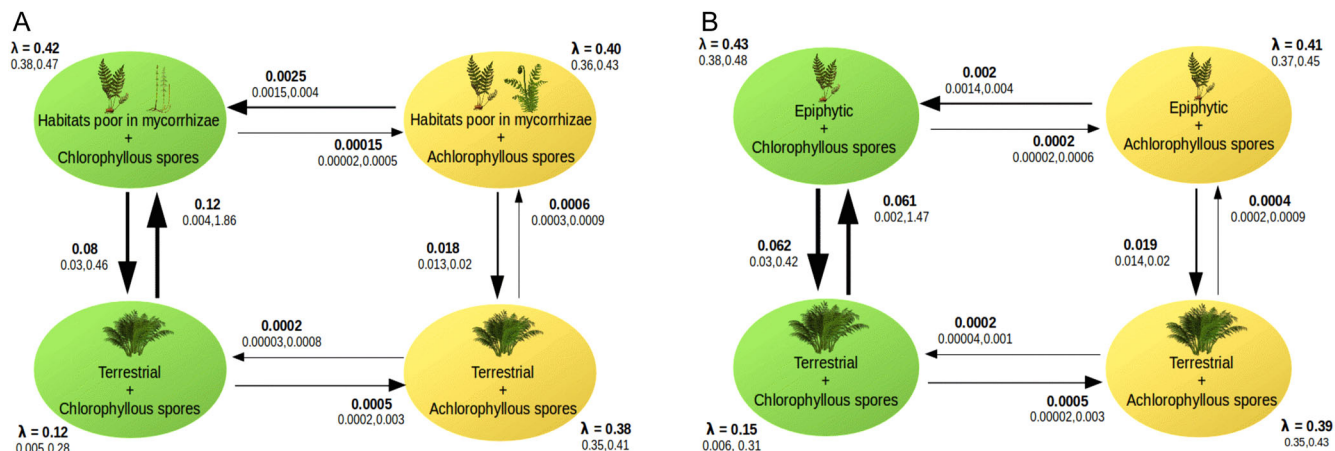


FIGURE 4 Estimation of transition rates of correlated evolution models using a multistate speciation and extinction analysis (MuSSE). (A) MuSSE analysis of chlorophyllous-spored species and habitats poor in mycorrhizae (epiphytic and waterlogged habitats). Transition and speciation rates (λ) (events per millions of years) correspond to the median of the posterior probabilities. The 95% confidence intervals are reported below each value. Extinction rate (μ) = 0.36 (0.33, 0.39). (B) MuSSE analysis of chlorophyllous-spored species and epiphytic and terrestrial habitats (excluding waterlogged species). Transition and speciation rates (λ) (events per millions of years) correspond to the median of the posterior probabilities. 95% confidence intervals are reported below each value. Extinction rates for all character states is $\mu = 0.37$ (0.33, 0.41). Images from [pngwing.com](https://www.pngwing.com).

TABLE 10 Set of multistate models fitted for the combination of habitat (poor in mycorrhizae vs. terrestrial; epiphytes vs. terrestrial) and spore type (chlorophyllous vs. achlorophyllous). In all models, the extinction rate was constrained to be equal for all states. AIC: Akaike information criterion; dAIC: delta AIC; AICw: AIC weight.

Model	AIC	dAIC	AICw
Habitats poor in mycorrhizae vs. terrestrial			
dull. null	16028.44	635.94	8.06E-139
MuSSE	15968.18	575.68	9.82E-126
MuHISSE dep	15498.67	106.17	8.8E-24
MuHISSE ind	15510.44	117.94	2.45E-26
MuCID4	15431.78	39.28	2.9E-09
MuCID6	15404.68	12.18	0.0023
MuCID8	15392.5	0	0.99
Epiphytes vs. terrestrial			
dull. null	15573.44	600.62	3.77E-131
MuSSE	15517.4	544.58	5.56E-119
MuHISSE dep	15056.08	83.26	8.3E-19
MuHISSE ind	15089.83	117.01	3.89E-26
MuCID4	15010.31	37.49	7.2E-09
MuCID6	14984.66	11.84	0.0027
MuCID8	14972.82	0	0.99

significant relationships shown by the χ^2 tests and the phylogenetic logistic regressions. Although we used the most comprehensive dataset of its type available, the relatively small number of species ($N = 419$) with

information on spore type, mycorrhizal associations, and phylogeny likely limits our ability to draw robust inferences. Moreover, here we accounted for mycorrhizal associations as the range of different fungal partners in ferns, although different fungal groups may not be ecologically equivalent. For instance, dark septate endophytes are more common in epiphytic habitats than arbuscular mycorrhizae, and recently it has been reported that associations with fungi from the class Mucoromycotina are more common in the roots of pteridophytes living in wetlands than arbuscular fungi (Perez-Lamarque et al., 2022). Empirical and theoretical studies are needed to fill the gaps on our knowledge of mycorrhizae and their influence on spore germination and fern ecology.

The paucity of mycorrhizal fungi in epiphytic and waterlogged habitats (Brundrett and Tedersoo, 2018) is putatively explained, respectively, by the poor aerial dispersal of arbuscular mycorrhizal spores into the canopy (Willis et al., 2013) and anoxic conditions in waterlogged soils (Helgason and Fitter, 2009). These previous observations are in line with our findings of correlated evolution between mycorrhizal associations and these habitats supporting our hypothesis H4 (Tables 7 and 8). The facultative associations of plants with mycorrhizal fungi, their ability to switch fungal partners, and/or the independence of fungal associations have been recognized as key processes in the colonization and adaptation to novel habitats (Rasmussen, 1995; Martos et al., 2012). For example, although the limited nutrient content of orchid seeds makes them highly dependent on mycorrhizae for germination (Rasmussen, 1995; Martos et al., 2012), 75% of them inhabit the forest canopy (Zotz et al., 2021). One reason for their success as epiphytes is the ability to recruit free-living saprotrophic fungi (Yukawa et al., 2009). Similarly,

sporophytes of epiphytic grammitid ferns can associate with ascomycetes (Lehnert et al., 2009, 2017) and, moreover, their spores and gametophytes can be cultured without mycorrhizal associations (Stokey and Atkinson, 1958). Facultative mycorrhizal associations have been reported in *Equisetum* with individuals in wet habitats generally lacking such associations, whereas individuals from well-drained soils generally have mycorrhizae (Dhillion, 1993; Lehnert et al., 2017). Similar infraspecific variation has been reported in epiphytic species of *Hymenophyllum*, where individuals that inhabit lower zones of a tree may have mycorrhizal associations, whereas plants growing in higher zones lack any association (Lehnert et al., 2017). For liverworts with CS such as *Plagiochila*, Kottke and Nebel (2005) suggested that the independence of fungal partners facilitates the colonization of the canopy. Together, these previous studies suggest that fern species with fungal-free autotrophic spores and gametophytes could have been advantageous during their dispersal and/or radiation into habitats with low mycorrhizal availability such as forest canopies and oceanic islands.

Consistent with this interpretation, transition rates show that CS in ferns evolved in association with habitats poor in mycorrhizae (H5). Furthermore, epiphytic lineages were more likely to shift from AS to CS rather than in the opposite direction (Figures 3B and 4B), and lineages with CS evolved at higher rates in epiphytic and waterlogged habitats than lineages with AS did (Figure 4A and B). All these results document the evolutionary opportunities presented to ferns with CS in habitats poor in mycorrhizal partners. However, our MuHISSE models suggest that traits other than our focal traits influenced the diversification of ferns as well. This finding is in line with the results of previous studies with Polypodiaceae, in which epiphytic lineages with CS and noncordiform gametophytes were found to have higher diversification rates when compared with lineages with other traits (Schneider et al., 2004; Sundue et al., 2015). Therefore, the success of ferns with CS in novel habitats such as the forest canopy is related to several other traits found in these species. However, considering that fern populations can only colonize sites where their spores survive, spore characteristics should play a key role in the conquest of the canopy. One such key characteristic of CS is their lack of dormancy (Lloyd and Klekowski, 1970; Sundue et al., 2015). Unlike AS, CS germinate immediately upon arrival on the substrate regardless of environmental conditions, and furthermore, in some species of grammitid ferns and Hymenophyllaceae, germination within the sporangium has been reported (Stokey, 1940; Stokey and Atkinson, 1958; Lloyd and Klekowski, 1970; Yoro, 1971; Sundue, 2010). The lack of dormancy and faster germination of CS has been called a key trait in tropical epiphytic bryophytes for the short-distance dispersal and maintenance of populations in suitable microhabitats in the canopy (van Zanten and Gradstein, 1988; Kürschner, 2004). In seed plants, nondormancy is considered a trait associated with high dispersal and colonization ability, although it exposes seedlings to potentially unsuitable environmental conditions,

so that many species have evolved dormancy in response (Willis et al., 2014). We evaluated the number of transitions toward CS in the fern phylogeny and found that this character evolved approximately 23 times (24 changes between states on average; Appendix S1) in our fern phylogeny. Consequently, we suspect that ferns with CS may have been less affected by their nondormancy due to the constantly humid conditions in tropical cloud forests where the majority of fern species occur (Kreft et al., 2010; Kessler et al., 2011; Taylor et al., 2022).

CONCLUSIONS

Despite the evident ecological differences between CS and AS, the CS trait has rarely been considered in the context of fern biogeography and evolution. Here, we tested the novel hypothesis that CS represent an adaptation to habitats where mycorrhizal fungi are absent or infrequent. We found that CS are indeed overrepresented among fern species occurring in epiphytic and waterlogged habitats where such fungi are infrequent and that CS have fewer associations with mycorrhizae. Our analyses show that spore type evolved along with the habitats occupied by ferns and that transition rates toward habitats that are poor in mycorrhizae are higher for lineages with CS. Although the evolution of epiphytism in ferns cannot be explained by a single trait, we suggest that the CS was probably key for the conquest of novel habitats. However, it is important to bear in mind that our results are ultimately correlative and that showing a causal relationship will require experimental studies, e.g., by testing whether fern species with CS not only germinate more quickly, but also grow faster and have higher survival rates on soils without mycorrhizae than do species with AS.

AUTHOR CONTRIBUTIONS

D.M.M. and M.K. conceived the study; D.M.M., M.K., and G.Z. provided data; D.M.M., M.K., W.T., M.C., and M.S. designed the data analyses; D.M.M. performed data analyses; all authors contributed to the interpretation of the data; D.M.M. and M.K. wrote the original draft with input from H.K., W.T., G.Z., M.S., and M.C.


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DATA AVAILABILITY STATEMENT

The phylogenetic tree, a database on traits (habit, spore type), and R scripts used in this paper are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.bnzs7h4cr> (Mellado-Mansilla et al., 2022).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Supporting information on species richness per state, phylogenetic logistic regressions, and stochastic mapping of the spore type.

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