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MACROEVOLUTIONARY PERSPECTIVES ON BIOTIC INTERACTIONS

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Does evolutionary history determine specificity in broad ecological interactions?

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Abstract

1. Most species engage in broad interactions, in which they interact with multiple partner species. The evolutionary processes that generate such generalized interactions remain unknown, partly due to the difficulty in comparing their breadth.
2. We argue that the interaction specificity of species involved in broad interactions evolves in three ways: (a) *assemblage specialization*, in which a species specializes on particular host species that contribute unique resources, yielding specialization on the entire host assemblage; (b) *apparent generalism*, in which a species specializes on one or few host species that contribute unique resources, but also associates with other host species that contribute functionally redundant resources; and (c) *true generalism*, in which a species associates with multiple hosts that overlap functionally, and that are geographically interchangeable based on opportunity for encounter, leading to frequent host switching.
3. We performed a phylogenetically controlled analysis of data on mycorrhizal fungal associations for approximately 25% of the orchid subfamily Cypripedioideae to determine whether these plants have specialized on their mycorrhizal fungal communities, or whether they are true generalists. We also assessed the impact of environmental factors on these associations.
4. Our results suggested strong support for apparent generalism, suggesting strong specialization on particular, dominant fungi and weak specialization on others. Large orchid clades associated with dominant fungal species, notably *Tulasnella cystidiophora* for genus *Cypripedium*, and *T. cystidiophora* and *T. calospora* for genus *Paphiopedilum*. Significant phylogenetic signal in fungal species richness per plant species, but not in the fungal phylogenetic diversity per plant species nor in the composition of fungal assemblages across orchid species suggested that plant phylogeny is an important determinant of fungal association. Mixed linear models showed that environment influenced specificity across plant species, and that observed differences were strongly driven by differences in sampling effort.

5. *Synthesis*. We found evidence of specialization of plant species on dominant fungal species, and to a lesser extent on their close relatives. The strong dominance of particular fungal species in these associations suggests important ecological roles for them, while environmental gradients in specificity suggest strong environmental filtering of these interactions.

KEYWORDS
cophylogeny, evolutionary ecology, mycorrhiza, Orchidaceae, plant-soil (below-ground) interactions, symbiosis, *Tulasnella*

1 | INTRODUCTION

The breadth of partners that a species associates with in an ecological interaction, called its *specificity*, depends both on the degree of specialization between the interacting species and on the opportunity for them to interact (Combes, 2004). Specificity ranges from the narrowest possible, in which a focal species interacts with only one host species (to avoid confusion, we hereafter refer to species interacting with the focal species as "host species"), to highly generalized, in which it will interact with many hosts. The most specialized parasitic interactions have long fascinated evolutionary biologists because parasites that obligately depend on their hosts have evolutionary histories that can parallel that of their hosts (Fahrenholz, 1913; von Ihering, 1891). In contrast, broad, or generalized, interactions do not evolve in parallel and so do not produce patterns of cophylogeny (Charleston & Perkins, 2003). This suggests that specificity in generalist focal species may be determined predominantly by the ecological opportunity to interact, rather than on adaptive specialization on any particular hosts.

The seemingly diffuse nature of broad interactions does not mean that evolutionary specialization does not occur (Brooks & McLennan, 1991; Page, 2003). Critical interactions in the life history of most, if not all, species involve multiple partners, with even the most specialized species generally having other interactions in which they are more generalized (Borowicz & Juliano, 1991; Cook & Rasplus, 2003; Hoeksema, 1999). For a given focal species, the extent of evolutionary specialization on a host species likely depends on the degree to which interacting with that host species fulfils a requirement of its niche (Tedersoo, Mett, Ishida, & Bahram, 2013), and may also depend on whether other potential hosts overlap in their ability to fill that niche. We call *true generalism* the case in which interaction with hosts is required, but host species can interchangeably fulfil the niche requirements of the focal species. This generates little or no natural selection maintaining the interaction with any particular host species, although there may be natural selection to maintain the interaction in general.

True generalism stands in contrast with apparent generalism and assemblage specialization. *Apparent generalism* occurs when a particular host fills some fundamental need that other host species do not fill, but other hosts without unique influences on the focal species have supplementary effects that may or may not be required. This situation should lead to specialization on the dominant host, but not necessarily on others, and so the association will appear to be broad. Finally, if all host species are uniquely important, then partner species may exhibit *assemblage specialization*, in which the focal species evolves to specialize on each of its host species, each of which incrementally fulfils the life-history requirements of the focal species. In the most extreme cases, assemblage specialization may yield phylosymbiosis, in which the phylogenies of hosts and focal species resemble each other while still allowing for multiple hosts per species (Brooks, Kohl, Brucker, Opstal, & Bordenstein, 2016).

True generalism, apparent generalism, and assemblage specialization should all yield distinct patterns with respect to the phylogeny of a clade of focal species. At one extreme, true generalism should lead to common host switching among closely related focal species, with the identity of hosts determined mostly by ecological opportunity rather than by biological filtering arising from compatibility. Theoretically, true generalism could generate a pattern in which all focal species in the clade uniformly interact with the same broad array of compatible hosts, although there are biogeographic and ecological constraints on the ranges of both partners that causes focal species to switch between locally available subsets of compatible hosts. Thus, the identities of host species in an interaction should differ among focal species without respect to evolutionary relationships, yielding no phylogenetic signal in host assemblages among partner species, and should more likely reflect the geographic ranges of their hosts. Apparent generalism should yield stabilizing selection for one or a few particular host species, with host switching among other co-occurring hosts common. If the ecological niche of a host species is relatively conserved across phylogeny, then this should result in phylogenetic signal for associations with the predominant hosts, but not for the remaining host clades in the assemblage. Finally, assemblage specialization should lead to strong phylogenetic signal in host assemblages among partner species, as natural selection maintaining the interaction should make interactions with close relatives of the host species more likely to occur than interactions with more distant relatives.

Although specificity is often used as a measure of specialization, it is actually determined by the biological compatibility of partners as well as their biogeographic range limits, which often depend on the

environment. Both focal species and their hosts have ranges limited by geological boundaries (Slatyer, Hirst, & Sexton, 2013) as well as by the past and current presence of appropriate partners (Poulin, Krasnov, & Mouillot, 2011). A focal species may experience selection for broader specificity, i.e. for more host species to interact with, if it specializes on specific hosts with narrow ranges. In such a case, the most likely types of host switches will involve new hosts that are closely related to the original host species and thus may provide a similar array of specialized partner services (Tedersoo et al., 2013). This pattern of specialization on relatives should yield phylogenetic signal in the host assemblage itself (assemblage specialization), or in the dominant hosts associated with (apparent generalism). Theoretically, climate change disrupting biotic interactions might also yield selection for broader specificity as the ranges of focal species and original hosts move apart (Memmott, Craze, Waser, & Price, 2007).

Environmental factors may also determine which hosts a focal species interacts with. Under true generalism, the environmental niche of the focal species is likely quite different from that of any of its host species. We expect that this should lead to patterns in which the environmental factors determining the geographic ranges of host species also strongly determine the set of hosts. For example, arbuscular mycorrhizal plants typically associate with several to many species of arbuscular mycorrhizal fungi, and these fungal assemblages generally become more diverse with increasing soil pH and precipitation, and less diverse with increasing elevation (Geml, 2017). Conversely, assemblage specialization should limit the range of the focal species to that of the hosts, and thus eliminate any possibility of statistical correlation between the breadth of the interaction and environmental factors. Under apparent generalism, host switches still occur and so the strength of the impact of environmental factors on the interaction likely depends on the number of hosts and whether they share some common range determinants. At the broadest spatial scale, specificity may also differ with latitude. The number of species of soil fungi decreases and the range size of fungal species increases with increasing latitude (Stevens, 1989; Tedersoo et al., 2014), making specialization more likely at high latitudes where fewer host species are likely to exist. Thus, specificity should become narrower with increasing distance from the equator. This latter situation would likely suggest apparent generalism in a clade of focal species, since higher latitude species and populations would appear more specialized than those at lower latitudes.

Here, we assess the influences of evolutionary history and environmental constraints on the specificity of ecological interactions. We focus on the orchid mycorrhiza, which is a relatively specialized interaction that nonetheless exhibits strong variability in the degree of specificity within orchid species. The mycorrhiza is a relatively generalized interaction in many plant clades, as a single plant species can often associate with many fungal species across different families and even different divisions (Molina, Massicotte, & Trappe, 1992). In orchids, this interaction becomes more specialized and involves the unusual directional movement of carbon from fungus to plant at least during the earliest stages of development (Bidartondo, 2005). This mycorrhiza is an obligate interaction with variable specificity, and studies suggest that, unlike most mycorrhizal plants, orchids may be limited in their ranges by their mycorrhizal specificity (Swarts, Sinclair, Francis, & Dixon, 2010). We studied the lady's slipper subfamily of the orchid family (Cypripedioideae, Orchidaceae) because it includes species that vary in their degree of mycorrhizal specificity, from several that are highly specialized on single fungal species to others that have highly generalized interactions with fungi across the Kingdom Fungi (Shefferson et al., 2007). We tested our three mutually exclusive hypotheses for the evolution of interaction specificity. At one extreme, specialization on a set of unique fungi and their close relatives would lead to strong phylogenetic signal in the set of mycorrhizal fungi that each plant species associates with, and little impact of environmental factors (assemblage specialization). At the other extreme, the need for some of a group of functionally redundant fungi would yield no phylogenetic signal in the set of mycorrhizal fungi, and a strong impact of environmental factors (true generalism). Apparent generalism would likely yield an intermediate signal, with both phylogenetic signal in the suite of mycorrhizal fungi, and environmental correlates.

2 | MATERIALS AND METHODS

2.1 | Dataset development and field methods

We created a dataset of mycorrhizal and other root-endophytic fungi for as many species as possible in the orchid subfamily Cypripedioideae. Our dataset included mycorrhizal information for 41 orchid species throughout the subfamily, approximately 25% of the roughly 160 Cypripedioideae species (Pridgeon, Cribb, Chase, & Rasmussen, 1999), and including 24 *Cypripedium* spp., 16 *Paphiopedilum* spp., and 1 *Phragmipedium* sp. (the dataset lacked representatives of the genera *Selenipedium* and *Mexipedium*). For purposes of comparison, we also found mycorrhizal information for four species of the subfamily Apostasioideae, which we used as an outgroup (Li et al., 2011; Unruh et al., 2018).

We constructed this dataset by combining mycorrhizal data gathered from the literature with mycorrhizal data that we generated from our own field studies. We found 16 studies documenting mycorrhizal fungi for species across the subfamily (Table S1), from sites throughout the Northern Hemisphere (Figure S1), as well as two studies on the Apostasioideae (Kristiansen, Freudenstein, Rasmussen, & Rasmussen, 2004; Yukawa, Ogura-Tsujita, Shefferson, & Yokoyama, 2009). We gathered the taxonomic identities of all putatively mycorrhizal fungal species noted as originating from peloton-containing root tissue in these studies, the orchid species from which they were identified, and, when possible, which fungi were found in which plant individuals. We supplemented this with data from mycorrhizal samples that we collected from 2007 to 2016. We collected root samples from 14 populations of 12 *Cypripedium* spp., one population of *Paphiopedilum dianthum*, and three populations of *Phragmipedium longifolium* covering sites in China, Russia, and the United States (Figure S1, Table S1). Including data gathered from

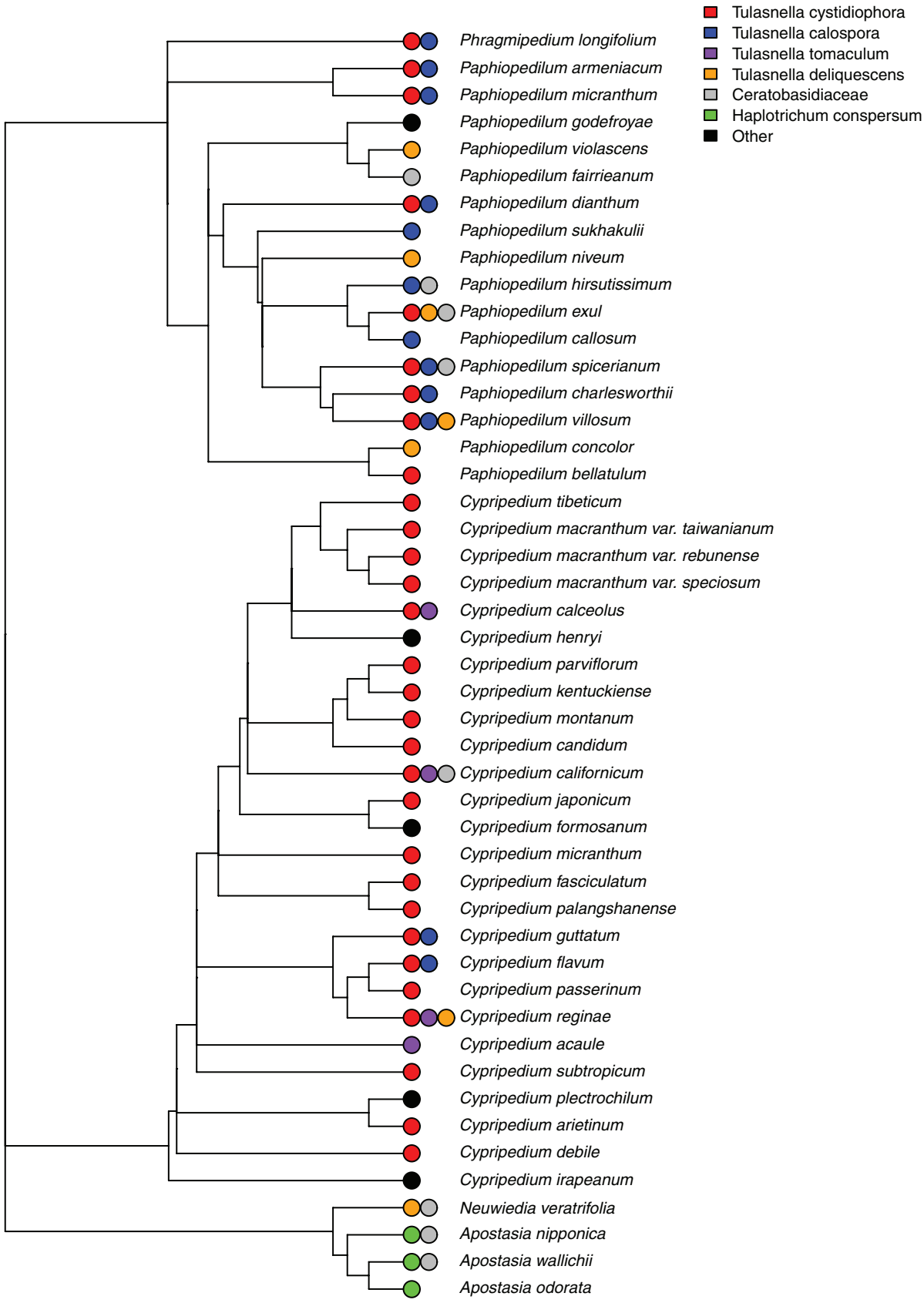

FIGURE 1 Dominant fungal hosts for each plant species plotted on a three-gene phylogeny of the sampled species of Cypripedioideae used in this analysis, with particular focus on *Tulasnella cystidiophora*, *T. calospora*, *T. deliquescens*, *T. tomaculum*, *Haplotrichum conspersum*, and members of the Ceratobasidiaceae

the literature, we included mycorrhizal data from 147 populations from throughout the subfamily, plus a further 11 populations of the Apostasioideae.

2.2 | Fungal identification

Our sampling protocol involved collecting 1–4 roots each from 1 to 10 individuals per population, depending on the number of individuals in the population and the size of each plant. These were put on ice, taken to the lab, washed and surface-sterilized. Sections at 0.5 cm intervals were observed under a compound microscope for the presence of pelotons. All peloton-containing sections were then used for fungal identification. We also collected 1–4 leaves from each population for use in plant phylogeny development.

We extracted bulk DNA from root samples displaying orchid mycorrhizal morphology. We then PCR amplified the *ITS* (internal transcribed spacer) and *mtLSU* (mitochondrial large subunit) of these samples. Amplified samples were subject to 3–4 enzyme RFLP analysis, and representative samples were Sanger sequenced. Sequences were compared to existing barcode sequences on Genbank using BLAST. We identified species when *ITS* sequences aligned with identified accessions on Genbank with 100% identity. We assigned species identity to *ITS* sequences that did not perfectly match existing accessions using *Emerencia.org* (Nilsson, Kristiansson, Ryberg, & Larsson, 2005). Our *mtLSU* sequences were not specific enough to provide species-level identification, but were used to assign broader classes and, in most cases, confirmed *ITS*-based classifications. Species known to form mycorrhizal associations were marked as mycorrhizal in our database, while species known to engage in non-mycorrhizal interactions, with morphologies suggesting an inability to form a mycorrhiza, or with unknown identity were assumed not to be mycorrhizal. Further details on laboratory and analytical methods related to fungal identification are provided in the Supplementary Methods.

2.3 | Fungal and plant phylogeny development

We developed a phylogeny of all fungi mycorrhizal with Cypripedioideae species in our mycorrhizal database (Figure S2). Unlike other assessments of fungal specificity in orchids, we did not limit this phylogeny to any particular group of fungi, instead including all identified fungi mycorrhizal with this group. To this end, we used the Open Tree of Life as a backbone for a supertree including all relevant fungi (Michonneau, Brown, & Winter, 2016; R Core Team, 2018). The Open Tree of Life included numerous polytomies, particularly for the fungal families Tulasnellaceae and Ceratobasidiaceae, and the genus *Russula*, which are dominant partners of Cypripedioideae species. So, we replaced those clades with phylogenies developed either by ourselves or others. Full details of the creation of these trees and the resulting supertree are provided in the Supplemental Methods.

We developed a supertree of Cypripedioideae species sampled in this study (Figure 1). Although species in the Cypripedioideae are represented in the Open Tree of Life, species were not resolved within genera at the time of writing. Instead, we used the general phylogeny of the Orchidaceae and of genera in the Cypripedioideae presented in Unruh et al. (2018) as a backbone. Onto this we added the phylogeny of genus *Cypripedium* developed in Li et al. (2011), and phylogenies that we developed of sampled *Paphiopedilum* species and Apostasioideae species. Full details are provided in the Supplemental Methods.

2.4 | Environmental analyses of specificity

We tested the assemblage specialization hypothesis by analysing phylogenetic patterns in mycorrhizal specificity and the composition of mycorrhizal assemblages, as well as identifying environmental correlates to these patterns. First, we assessed the specificity of the interaction at both the plant population and the plant species levels. We measured specificity as the species richness (SR) and the phylogenetic diversity (PD) of mycorrhizal fungi interacting with them. An orchid species' fungal PD was estimated as the sum of branch lengths in the most parsimonious subtree of our fungal supertree composed of only the fungi mycorrhizal with that plant species. Both SR and PD are relatively low when specificity is narrow, suggesting specialization, and relatively high when specificity is broad, suggesting generalism.

We hypothesized that plant and fungal partners may change due to environmental differences across sites. We explored these relationships with a non-metric multidimensional scaling (NMDS) of plant and fungal presence against all 19 bioclimatic variables extrapolated for each site from WorldClim at the 1 km scale (Fick & Hijmans, 2017). In total, this yielded climatic data for 153 populations with geographic data. NMDS was conducted in R 3.5.2 (R Core Team, 2018).

Next, we assessed the determinants of overall mycorrhizal specificity within plant populations by using these terms as responses in generalized linear mixed models. Fixed factors included five key climatic variables identified from our NMDS analyses as uniquely determining plant and fungal presence with an R^2 above 0.75, absolute value of latitude (hereafter, absolute latitude), and the number of individuals sampled. Four sets of mixed models were developed with different random factors used. In the first set, random factors included species, and distance from the prime meridian nested within continent (this latter term was meant to account for environmental variation unaccounted for by our WorldClim variables, and was thought a better metric than longitude since the distance between longitudinal meridians decreases with increasing latitude). The second set included species and absolute latitude nested within continent as random factors, the third set included continent and species as random factors, and the fourth set included only species as a random factor. We did not include evolutionary history in these models, instead using other methods to investigate the role of phylogeny (described later). All mixed models were performed using package *lme4* for R 3.5.2 (Bates, Maechler, Bolker, & Walker, 2015; R Core Team, 2018), and we conducted exhaustive model selection using minimum

AIC_c as the criterion for the best-fit model, via the *dredge* function in package *MuMIn* (Bartoń, 2014).

Finally, we tested the impact of environment and sampling effort on specificity at the plant species level. Here, we constructed global generalized linear models in which fungal species richness (Poisson) or fungal phylogenetic diversity (Gaussian) were determined by the latitudinal centre of populations, central distance from the prime meridian of populations, the maximum distance between all pairs of populations, the mean number of individuals sampled per population, the number of populations, the mean fungal diversity per population (species richness or phylogenetic diversity, respectively), the 5 WorldClim variables used before, the interaction of the number of populations and mean number of individuals per population sampled, and the interaction of the number of populations and the mean fungal diversity per population. Model selection was conducted as before. In both the population- and species-level analyses, significant environmental or geographic factors in the best-fit models would support apparent or true generalism.

Finally, we assessed the potential for our phylogenetic analyses to be biased by uneven sampling across taxa. We used the best-fit models predicting fungal specificity in plant species to estimate the species richness and phylogenetic diversity of plant species under even sampling effort (set at 10 populations per species, with 10 individuals per population).

2.5 | Phylogenetic signal and evolutionary history of specificity

We reconstructed the evolutionary history of species-level mycorrhizal specificity on the plant phylogeny using *fastAnc* in package *phytools* in R 3.5.2 (R Core Team, 2018; Revell, 2012). We assessed mycorrhizal specificity as the estimated fungal species richness and phylogenetic diversity of mycorrhizal fungal species per plant species under even sampling effort, and estimated Pagel's λ for this trait. Although other metrics are widely used to measure phylogenetic signal (e.g. Blomberg's *K*), we used Pagel's λ because it is the only metric unbiased by the number of OTUs in the phylogeny (Münkemüller et al., 2012). We compared our estimated λ for both estimated fungal species richness and observed phylogenetic diversity against 1,000 bootstraps, in which trait values were randomly shuffled on the phylogeny. Significantly large λ would indicate phylogenetic signal, meaning that more closely-related species exhibit more similar values of specificity. We expected that assemblage specialization would lead to strong phylogenetic signal in specificity, with most orchids having very few mycorrhizal fungi, while true generalism should not lead to phylogenetic signal.

Although assemblage specialization should lead to phylogenetic signal in specificity itself, apparent generalism may or may not. Next, we estimated the weighted, standardized Unifrac distance among fungal communities associated with each orchid species (Lozupone & Knight, 2005), and created a matrix of these values. We performed a Mantel test of these values against the phylogenetic distance between each pair of plant species, where assemblage specialization would be supported by a significant positive correlation, and apparent generalism and true generalism would be supported without such a correlation.

Apparent generalism, in which a dominant host (here, mycorrhizal fungal species) exists and all other hosts are less important, was tested against true generalism by identifying the most dominant mycorrhizal fungal species for each plant species, and mapping these dominant associations onto our plant phylogeny. Dominant mycorrhizal fungi were fungal species associated with >1 plant individual of each plant species, and the most dominant fungi were the top-ranked fungi or the top two fungi in cases where two fungi were very frequent. Apparent generalism predicts that different orchid clades will exhibit strong preferences for particular fungal species within the mycorrhizal assemblage, making close relatives share the same or closely related dominant mycorrhizal fungal species, while true generalism predicts no such pattern.

3 | RESULTS

3.1 | Mycorrhizal fungi identified

Plants in the Cypripedioideae associated with fungi from 18 fungal families, although some unidentified fungal species may have belonged to families outside of these. These families included the Botryobasidiaceae, Ceratobasidiaceae, Clavariaceae, Corticiaceae, Entolomataceae, Glomeraceae, Hydnaceae, Hygrophoraceae, Inocybaceae, Leotiaceae, Pluteaceae, Russulaceae, Sebacinaceae, Serendipitaceae, Thelephoraceae, Tricholomataceae, Tulasnellaceae and Vibrisseaceae.

The dominant mycorrhizal fungal species used by orchid species differed among plant genera (Figure 1). We identified 19 fungal species that occurred in more than one plant individual each, which we defined as dominant fungi. These included *Ceratobasidium cornigerum*, *Haplotrichum conspersum*, *Leptodontidium orchidicola*, *Pezoloma ericae*, *Rhizophagus clarus*, *Russula crustosa*, *R. sardonia*, *Sebacina epigaea*, *Serendipita vermifera*, *Sistotrema brinkmannii*, *Thanatephorus ochraceus*, *Tomentella sublilacina*, *Tulasnella asymmetrica*, *T. calospora*, *T. cystidiophora*, *T. deliquescens*, *T. pruinosa*, *T. tomaculum* and *T. violea*. Of these, only the ascomycetes, *Leptodontidium orchidicola* and *Pezoloma ericae*, are endophytic species that are of unknown ecology. Most fungal species were rarely encountered, with a few particularly dominant. The most common fungal species was *Tulasnella cystidiophora*, which was found in 28 plant species (Figure 1; Table S1). The next most common was *T. calospora*, which was found in 12 plant species, followed by *T. deliquescens*, which was found in 5 plant species (Figure 1; Table S1).

3.2 | Environmental factors determining the presence of plant species

Species from the orchid genera *Cypripedium*, *Paphiopedilum* and *Phragmipedium* clustered in different climates (Figure 2). All climatic

variables exerted a significant influence in our NMDS analysis (all $p < 0.001$, with an overall stress value of 0.017 and a linear fit of $R^2 = 0.999$, both metrics suggesting excellent representation of the data), with temperature seasonality (bio4) and mean annual precipitation (bio12) accounting for particularly large shares of variation in NMDS space ($R^2 > 0.95$, Table S2). Visual assessment suggested overlap between precipitation variables, and between variables denoting temperature extremes (Table S2). Temperature seasonality (bio4), annual range in temperature (bio7), mean temperature in the coldest quarter (bio11), mean annual precipitation (bio12), and precipitation seasonality (bio15) stood out as five variables representing the extent of unique influences of climate in the first two NMDS coordinates.

3.3 | Mycorrhizal specificity

Specificity varied across orchid species, with an average specificity per plant species of 3.00 ± 0.41 fungal species (phylogenetic diversity of 14.61 ± 2.16), and a mode of two fungal species (Figure S3). 14 orchid species (31.1% of sampled Cypripedioideae species with mycorrhizal data) associated with only one fungal species, with a further 13 (28.9%) associating with only two fungi (Table S1). The set of fungal root endophytes was broadest in the orchid species *Cypripedium acaule*, *Cypripedium calceolus*, and *Phragmipedium longifolium*, which associated with 14, 12 and 10 fungal species (PD of 49.70, 59.32 and 42.70), respectively (Table S1). In genus *Paphiopedilum*, the set of fungal root endophytes was broadest in the orchid species *P. dianthum*, *P. hirsutissimum* and *P. villosum*, which each associated with five fungal species (PD of 27.90, 29.12 and 20.60, respectively; Table S1). At the genus level, *Cypripedium* associated with an average of 3.15 ± 0.65 fungal species (phylogenetic diversity of 15.79 ± 3.35), while *Paphiopedilum* associated with an average of 2.50 ± 0.37 (phylogenetic diversity of 12.51 ± 2.61). Specificity at the subfamily level did not differ significantly between Cypripedioideae and Apostasioideae (Cypripedioideae: 3.07 ± 0.45 fungal species, phylogenetic diversity of 15.19 ± 2.33; Apostasioideae: 2.00 ± 0.41 fungal species, phylogenetic diversity of 8.33 ± 3.01; Welch two-sample *t* test of species richness: $t_{13.15} = -1.77$, $p = 0.112$; Welch two-sample *t* test of phylogenetic diversity *t* test: $t_{7.49} = -1.80$, $p = 0.100$).

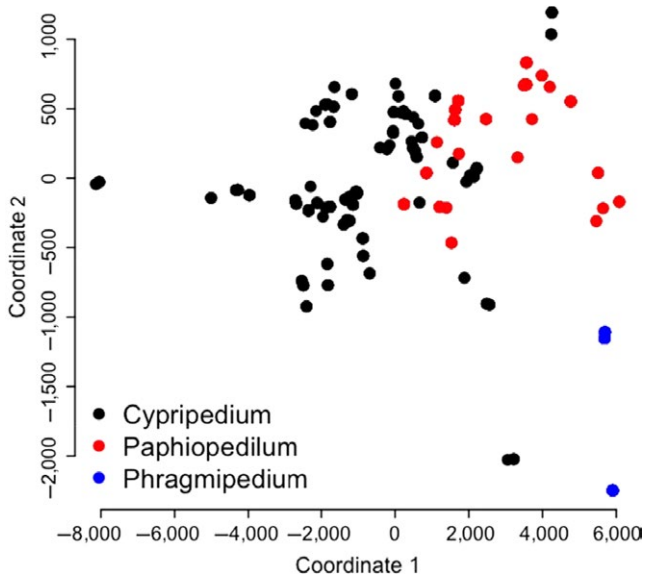

FIGURE 2 Non-metric multidimensional scaling (NMDS) biplot showing plant populations against climatic variables provided by WorldClim, in the plane of the first two NMDS axes

Analysis of the determinants of mycorrhizal specificity supported roles for both environment and sampling effort. At the plant population level, fungal species richness varied positively with mean temperature in the coldest quarter (bio11), the number of individuals sampled, continent and plant species (Figure 3; Table S3). Fungal phylogenetic diversity among plant populations varied positively with the number of individuals sampled, and by plant species and continent (Figure 3; Table S4). When potentially non-mycorrhizal endophytes were removed from the analysis, the best-fit model for fungal species richness was the same, but the best-fit model for fungal phylogenetic diversity also included the absolute value of latitude.

At the plant species level, fungal species richness varied positively with the number of populations and with within-plant-species mean fungal diversity across populations (Figure 4; Table S5). Fungal phylogenetic diversity varied negatively with temperature seasonality (bio4), and positively with the maximum distance between populations, the number of populations, the within-plant-species mean number of individuals sampled, and the within-plant-species mean fungal diversity across populations (Figure 4; Table S6). The best-fit models explained a large part of the variation in the dataset (SR best-fit model: pseudo-$R^2 = 0.785$; PD best-fit model: adjusted $R^2 = 0.866$). Removing potentially non-mycorrhizal endophytes yielded similar best-fit models, except that temperature seasonality was no longer an explanatory factor for fungal phylogenetic diversity. Thus, sampling effort strongly determines observed specificity, but the latter scales linearly with mean fungal diversity observed within populations, suggesting that trends noted at the population level can be used to predict trends at the species level. Environmental factors also exert an influence on the evolutionary breadth of fungi associated with, but not on fungal species richness (Figure 4).

Ancestral character reconstruction suggested that the common ancestor of these genera likely associated with many mycorrhizal fungi, but that these fungi were likely closely related. Measured as fungal species richness, specificity at the deepest nodes were predicted to be generalized, while when measured as phylogenetic diversity, specificity at the deepest nodes was predicted to be intermediate (Figure S4). Specialization occurred at many points in the evolutionary history of the Cypripedioideae, and when it did it involved a loss of fungal species and a narrowing of the phylogenetic breadth of fungi associated with (Figure S4). In contrast, the Apostasioideae appeared to specialize as a whole. There appeared to be no difference in proportion of the genera *Paphiopedilum* and

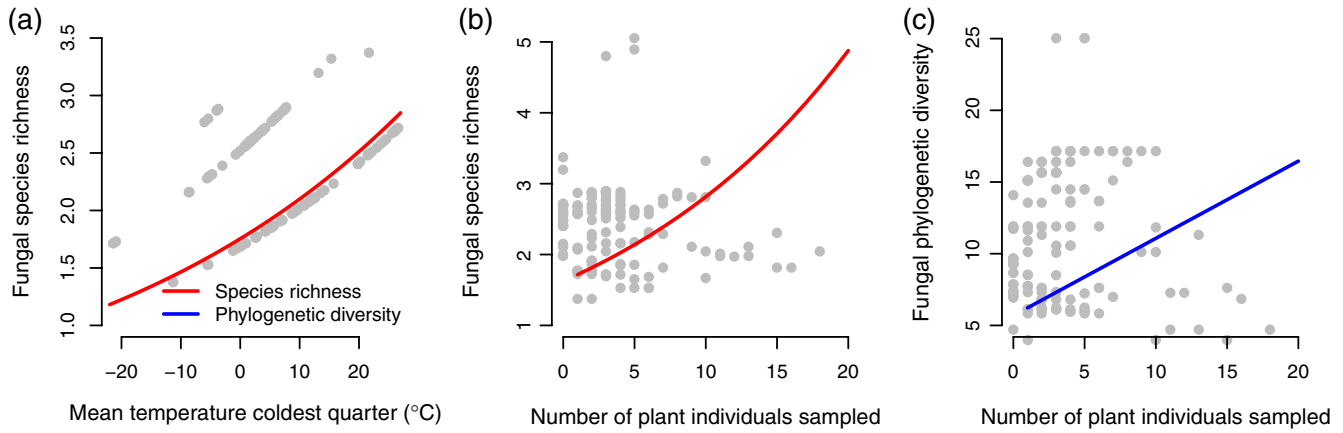

FIGURE 3 Impacts of tested factors on mycorrhizal specificity, given as either fungal species richness or fungal phylogenetic diversity, of plant populations included in this analysis. Factors presented are those retained within the associated best-fit models, including impacts of (a) mean temperature in °C during the coldest quarter of the year on fungal species richness, and number of plant individuals sampled on (b) fungal species richness and (c) fungal phylogenetic diversity. Grey dots are actual population-level data points

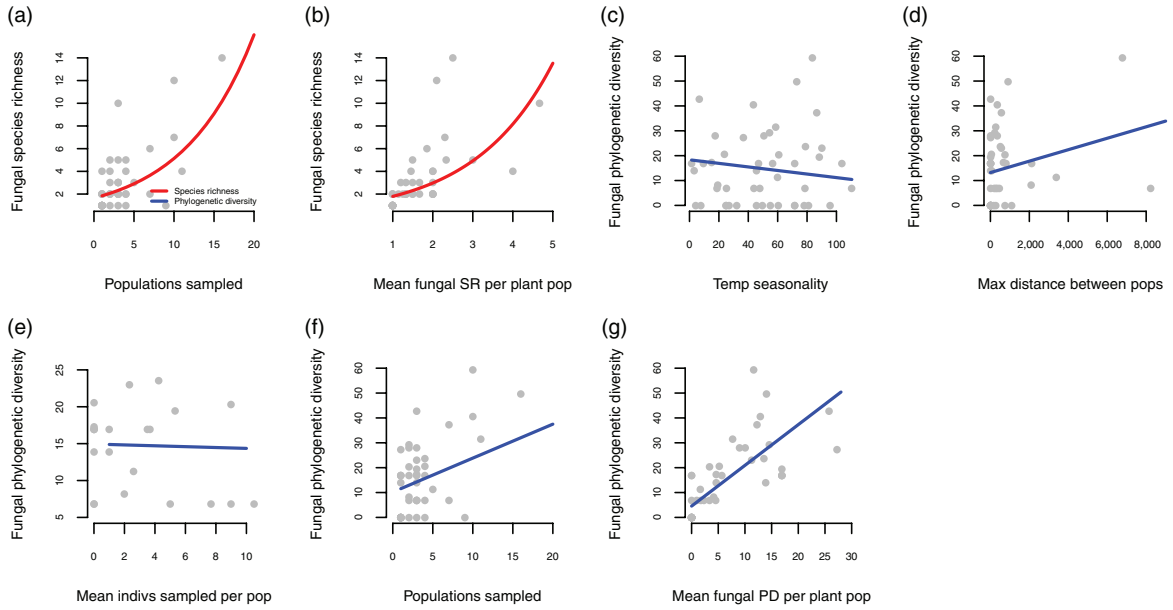

FIGURE 4 Impacts of tested factors on mycorrhizal specificity, given as either fungal species richness or fungal phylogenetic diversity, of plant species included in this analysis. Factors presented are those retained within the associated best-fit models, including effects of (a) number of populations per species and (b) mean population-level fungal species richness on species-level fungal species richness, (c) temperature seasonality (bio4, given as the standard deviation of mean monthly temperature), (d) maximum distance between conspecific populations, (e) mean number of individuals sampled per population, (f) number of populations per species, and (g) mean population-level fungal phylogenetic diversity on species-level fungal phylogenetic diversity. Grey dots represent actual species-level data points

Cypripedium that had evolved to be specialized versus generalist (Figure S4).

3.4 | Specialization versus generalism

Phylogenetic analysis of specificity supported apparent generalism in the Cypripedioideae. Dominant fungal species were retained in specific clades, as expected under this hypothesis. Notably, *Cypripedium* preferentially associated with the fungal species *Tulasnella cystidiophora* (Figure 1). *Paphiopedilum* associations were dominated by the fungi *T. calospora* and *T. cystidiophora*, and the Apostasioideae associated primarily with members of the fungal families Ceratobasidiaceae and Botryobasidiaceae (Figure 1). Though less common, *T. deliquescens* associated with scattered species throughout the Cypripedioideeae, and *T. tomaculum* was found to be exclusively associated with several species within genus *Cypripedium* (Figure 1). Fungal species richness for each plant species corrected for sampling effort exhibited significant phylogenetic signal (Pagel's

$\lambda = 0.999$, $p = 0.013$), while fungal phylogenetic diversity did not (Pagel's $\lambda = 6.61 \times 10^{-5}$, $p = 1.000$). Support against assemblage specialization is reinforced by the lack of a correlation between the phylogenetic distance between mycorrhizal fungal communities among plant species on the one hand, and the phylogenetic distance between those plant species on the other (Spearman rank Mantel test of weighted UniFrac distances vs. plant phylogenetic distance: $r = 0.005$, $p = 0.363$; Figure S5).

4 | DISCUSSION

Patterns in mycorrhizal specificity among orchid species in the Cypripedioideae supported the apparent generalism hypothesis. Thus, plants may typically require several fungi to meet their needs, and specialization occurs on a few dominant fungal species and to a lesser extent on their close relatives. In general, particular species of fungi dominated associations with particular clades of orchids, with genus *Cypripedium* associating most strongly with the fungal species *Tulasnella cystidiophora*, and genus *Paphiopedilum* dominated by both *T. cystidiophora* and *T. calospora* (Figure 1). The fungal diversity that each plant species associated with varied with a combination of factors, including plant phylogeny, environmental variables such as latitude and mean annual precipitation, fungal diversity encountered at the population level, and sampling effort itself (Figures 1, 3, 4, and Figure S4).

Geographic and environmental patterns in the distributions of plant clades and their mycorrhizal fungi suggest that specialization varies with environmental conditions. The primarily tropical East Asian distribution of *Paphiopedilum* contrasts strongly with the temperate Northern Hemisphere distribution of *Cypripedium*, and so may account for some of these differences. We also found increasing fungal species richness at the population level with increasing mean temperature in the coldest quarter, and decreasing fungal phylogenetic diversity at the plant-species level with temperature seasonality. Since this result was corrected for latitude, it suggests not that orchids in the Tropics have greater mycorrhizal diversity but that orchids occurring in warmer, less seasonally variable sites at equivalent latitudes have higher mycorrhizal diversity. This is consistent with studies noting that the diversity of saprotrophic, arbuscular and ectomycorrhizal fungi varies with temperature and other environmental factors (Allen et al., 1995; Geml, 2017; McGuire, Fierer, Bateman, Treseder, & Turner, 2012). These results differ from Oja et al. (2017), which noted small environmental influences on the suite of orchid mycorrhizal fungi associating with each of two orchid species, although the differences between studies may be due to differences in the spatial scales (microsite and regional in their study, vs. global in ours) and phylogenetic scales (two distantly related orchids in their study, vs. one large monophyletic subfamily in ours) explored. However, we cannot exclude environmentally driven biological filtering by plants resulting in this pattern. Such filtering might happen if plants alter their receptivity to different fungal species along environmental gradients (Querejeta, Egerton-Warburton, & Allen, 2009). For example, many ectomycorrhizal plants become less receptive to mycorrhizal fungi as plant-available nitrogen increases in the soil, leading to lower ectomycorrhizal fungal diversity along gradients of plant-available nitrogen (Lilleskov, Fahey, Horton, & Lovett, 2002). Regardless, climatic variables correlated with mycorrhizal specificity, reinforcing environmental factors as important filters on mycorrhizal interactions in these orchid species.

Among the most interesting patterns we observed is the dominance of two fungal species in these mycorrhizal associations. The selective benefits of association with *T. cystidiophora*, a fungal species found often interacting with *Cypripedium* spp. and *Paphiopedilum* spp., and *T. calospora*, another frequent orchid associate, are not clear. Many photosynthetic orchid species are capable of extracting fungal energy as adults, a condition referred to as partial mycoheterotrophy (Gebauer, Preiss, & Gebauer, 2016; Selosse, Charpin, & Not, 2017), and some species have evolved to utilize fungal carbon exclusively (Selosse, Bocayuva, Kasuya, & Courty, 2016; Selosse et al., 2017). These fungi are generally thought to act as saprotrophs in forest environments (Rasmussen, 1995; Roberts, 1999), although *Tulasnella asymmetrica* is a fungus known to be facultatively ectomycorrhizal and exploited by mycoheterotrophic liverworts, as well (Bidartondo, Bruns, Weiß, Sérgio, & Read, 2003; Oberwinkler, Cruz, & Suárez, 2017). Thus, the choice of fungus may relate to the ability of the plant to extract carbohydrate resources from the fungus, although little research exists to corroborate this hypothesis.

While we cannot be certain as to the exact reasons that any particular orchid species associates with any other fungus, two explanations present themselves. First, expansions in the number of hosts that a focal species associates with may allow habitat specialists to persist in habitats that may be disadvantageous to some host species, especially if host geographic range varies with changing climate and habitat. The orchid *Cypripedium californicum* may be an example. This species is one of the few *Cypripedium* spp. in which individuals regularly exist that do not associate with the fungus *Tulasnella cystidiophora*, instead associating with potentially many other fungal species, and it is strongly restricted to serpentine sites with flowing water in North America (Pridgeon et al., 1999). We suggest that this orchid species would likely be even more rare if it exhibited more specialized mycorrhizal associations. Second, jumps to potentially ectomycorrhizal hosts may increase opportunities for dispersal in some species, as might have happened in *Cypripedium acaule*. This species is possibly the most common *Cypripedium* species in North America, occurring in both relatively pristine conditions as well as in strongly human-affected woodlands (Pridgeon et al., 1999). The latter hypothesis may also explain the orchid *Phragmipedium longifolium*'s presence both on pristine volcanic slopes and in commonly grazed pastureland (Muñoz & Warner, 2007). Both of these hypotheses predict range expansion as a result of expansion in the suite of mycorrhizal fungi associated with, although the former hypothesis likely involves stronger natural selection within populations while the latter hypothesis may involve escape from natural selection via dispersal.

In the orchid mycorrhiza, ecological opportunity is determined not just by the presence of the fungus, but also by its density in the local environment (McCormick et al., 2012). This suggests that the breadth of mycorrhizal fungi that a plant species associates with may have to do with historical success at finding the right fungal species. In cases where density of appropriate mycorrhizal fungi is typically low, there may be a selective advantage to host jumping, or host expansion. The sensitivity of orchids to fungal density in the environment may even vary across species, since orchids occurring sympatrically often utilize different fungal species, suggesting niche partitioning (McCormick & Jacquemyn, 2014; Shefferson, Weiß, Kull, & Taylor, 2005).

Our work identifies phylogenetic signal in the dominant fungi determining mycorrhizal specificity in this monophyletic subfamily, and the importance of environmental drivers at the global scale. Our results differ from previous studies, particularly as others have noted stronger phylogenetic signal in mycorrhizal specificity as a whole (Jacquemyn et al., 2011; Shefferson et al., 2007). Interpreting phylogenetic signal properly has been difficult for at least three reasons. First, phylogenetic signal is difficult to link to specific mechanisms and processes. Phylogenetic signal can result from balancing selection in which the optimal value of a trait shifts slowly as lineages branch, but may also result from natural selection that fluctuates randomly with time, and also from genetic drift (Losos, 2008). Although our work supports phylogenetic signal in mycorrhizal associations, it does not support phylogenetic niche conservatism of entire assemblages, which would require unusually strong similarity in fungal association among species suggesting that small shifts in association would be strongly selected against (Losos, 2008). Thus, we can exclude strong balancing selection for mycorrhizal association as a mechanism yielding these patterns, but we cannot exclude any other hypothetical mechanism.

Second, the scale of investigation may influence the observation of phylogenetic signal or other patterns in trait evolution. Studies focused on a number of closely related species occurring in the same region may find different patterns than studies such as this, which attempt to deal with deeper phylogenetic patterns occurring at the global scale. Third, the resolution of the phylogenetic tree may influence the observation of phylogenetic signal artefactually because incomplete sampling can produce strong contrasts among clades if intermediate species are generally excluded (Münkemüller et al., 2012).

5 | CONCLUSIONS

We have determined that mycorrhizal interactions in the orchid subfamily Cypripedioideae yield evolutionary patterns supporting apparent generalism. Thus, evolutionary history strongly determines the breadth and suite of interactors in ecological associations. The most dramatic piece missing from this work is the clear identification of the ecology of the fungal species, including even their basic distributions, sensitivities to environmental factors, and to what extent that associate with other plants and fungi via other interactions. We call for more work on this as well as on the global distribution of mycorrhizal fungi. We also believe that future strides should be made to understand to what extent ecological interactions evolve independently of one another, since organisms typically in engage in multiple interactions at once. For example, does specialization on pollinators influence the breadth of mycorrhizal fungi associating with a clade of plant species? A great deal more data collection and integration across studies needs to occur to achieve this goal.

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AUTHORS' CONTRIBUTIONS

R.P.S. conceived of the project, carried out fieldwork and lab work, carried out the statistical analysis, and wrote the paper; W.B., C.C.C., Y.-I.L., T.R.K., T.Y. and J.D. carried out field and lab work and contributed to the writing; H.J. contributed field and lab work.

DATA ACCESSIBILITY

All data used in this study is available on GenBank (Fungal sequences accession numbers: MK161221-7, MK161229-45, MK161270 and MK564622-6; Plant sequence accession numbers: MK161064–MK161087, MK161098–MK161107 and MK161246–MK161269).

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SUPPORTING INFORMATION

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