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Radiation of mushroom-forming fungi correlates with novel modes of protecting sexual fruiting bodies

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ABSTRACT

The protection of vulnerable developing structures evolved repeatedly in terrestrial organisms and includes, among others, viviparity in animals and the seed in land plants. In mushroom-forming fungi (Agaricomycetes), sexual spores are born on fruiting bodies, the growth of which is a complex developmental process that is exposed to environmental factors (e.g., desiccation, fungivorous animals). Mushroom-forming fungi evolved a series of innovations in fruiting body protection, however, how these emerged is obscure, leaving the evolutionary principles of fruiting body development poorly known. Here, we show that developmental innovations that lead to the spore-producing surface (hymenophore) being enclosed in a protected environment display asymmetry in their evolution and are associated with increased diversification rates. 'Enclosed' development evolved convergently and became a dominant developmental type in several clades of mushrooms. This probably mirrors spore production benefits for species with protected fruiting body initials, by better coping with environmental factors. Our observations highlight new morphological traits associated with mushroom diversification that parallel the evolution of protection strategies in other organisms, such as viviparity or the seed in animals or plants, respectively, but in the context of spore development, highlighting the general importance of protecting vulnerable progeny across the tree of life.

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1. Introduction

Increasing reproductive efficiency is key for all organisms and has prompted the evolution of sophisticated mechanisms for protecting offspring. Diverse solutions evolved for protecting developing youth across the tree of life; all these share nursing and protective mechanisms that optimize the individual's nutritional investment per propagule. Examples include placentation (Roberts et al., 2016), viviparity and matrotrophy in animals (Blackburn, 1999) or the seed in embryophytes (Goldberg et al., 1994). Many such traits are considered key innovations that spurred diversification (e.g., viviparity in fishes, Helmstetter et al., 2016), have arisen convergently (e.g., viviparity occurred ~150 times in vertebrates (Blackburn, 2015)), or underscore the evolutionary success of clades

(e.g., seed plants, Westoby and Rice, 1982, but see Vamosi et al., 2018). However, we have limited knowledge about how general the role of offspring protection is outside animals and plants.

Fungi reproduce by sexual or asexual spores, which are born on specialized spore-producing cells. In mushroom-forming fungi (Agaricomycetes), these cells compact into a spore-producing surface, the hymenophore, in which meiosis and spore production take place. Spore can be considered as the offspring of a fungus, therefore its successful production and survival in the hymenophore are crucial. The hymenophore is exposed to environmental impacts (e.g., desiccation, precipitation, UV radiation), mycophagous animals and parasites. Accordingly, many strategies evolved to protect the hymenophore (e.g., Braga et al., 2015). One such solution is the development of complex fruiting bodies, which provide support, a physical barrier and chemical defense against external factors (Künzler, 2018) as well as facilitate spore dispersal (Dressaire et al., 2016). Physical protection comes in many forms, including hyphal sheaths that cover either the entire fruiting body initial (universal veil) or parts of it (partial veil), or developing the hymenophore and spores inside the fruiting body (in so-called gasteroid and secotioid

fungi). All these strategies are analogous to the protective mechanisms in animals or plants such as viviparity or seed development, because mushroom-forming fungi involve specialized tissues that enclose the hymenophore and the developing spores into a protected environment, and we hereafter refer to it as enclosed development.

Several key principles of the evolution of fruiting bodies have been uncovered recently. Phylogenetic comparative analyses confidently suggest that ancestral morphologies were crust-like (i.e., resupinate) and that these repeatedly gave rise to a series of more complex forms (Hibbett, 2004; Varga et al., 2019). The most complex fruiting bodies are called pileate-stipitate morphologies (mushrooms with cap and stalk), which evolved several times convergently and probably represent stable attractors in the morphospace (Hibbett, 2004). Further, the emergence of complex morphologies correlates with higher speciation rates and may be a major driver of lineage diversification in mushroom-forming fungi (Agaricomycetes) (Sánchez-García et al., 2020; Varga et al., 2019). However, beyond the broadest morphological types, we know little about what drives the evolution of fruiting body morphologies and how novel fruiting body traits impact speciation and extinction patterns. For example, it is not known what aspect of the pileate-stipitate morphology – protection of the hymenophore, increased efficiency of spore dispersal or yet other attributes – may have been the key innovation for mushroom-forming fungi. Further, there are several phylogenetically co-distributed morphological innovations, such as structured hymenophore surfaces, which could additively or in other ways influence diversification rates. However, the effect of such co-distributed, observed or unobserved traits has not been tested yet in sophisticated statistical frameworks, such as multi-trait or hidden trait speciation and extinction models.

Here, we investigate the evolution of enclosed development among mushroom-forming fungi using comparative phylogenetic analyses and a previously published phylogeny of 5284 species (Varga et al., 2019). We demonstrate that enclosed development evolved repeatedly in the Agaricomycetes and correlates with an increased net diversification rate of species. We further show that other, phylogenetically co-distributed innovations (complex hymenophores, the presence of a cap) also impact the net diversification rates, and that their effects are independent from those of enclosed development. Our results further extend our knowledge about the evolutionary advantages of the protection of developing progenies and exemplify evolutionary innovations among mushroom-forming fungi.

2. Material and methods

2.1. Character coding

2.1.1. Developmental types

The character state assignment was based on whether the developing hymenophore is open to the environment or insulated from it at some point during development (Fig. 1, Supplementary Data 1). In our default coding regime (referred to as 3ST), open development (state 0) was defined as the hymenophore being exposed to the environment from the earliest primordial stages. In semi-enclosed development (state 1), the hymenophore is covered by a faint veil only in the earliest primordial stages or the cap margin is attached to the stem but detaches before cap expansion. In enclosed development (state 2), the hymenophore is closed at least until the young fruiting body stage. We coded gasteroid/secotioid species as enclosed. Our character state assignments were based on 52 previous histological studies covering 94 genera, taxonomic literature about veil structures (Supplementary Data 1) and morphological descriptions following Reijnders 1983 and

Clémencon 2012 (also see Supplementary Methods). Because main morphologies of mushroom-forming fungi usually bear universal features (e.g., coralloid fungi always follow open development), the above-mentioned sources were sufficient to assign states to >5000 species.

To explore the results' robustness to coding, we developed four alternative character state coding regimes (Supplementary Data 1, Supplementary Methods). A coding regime was created where a fourth character state was assigned to species with sequestrate or gasteroid fruiting bodies (referred to as 4ST1). We created further two modified versions of the 4ST1 coding. First, character states for certain ambiguous taxa were changed (4ST2, see Supplementary Methods). Second, we re-coded all marasmoid fungi to state 0 (four states dataset 3, 4ST3) because certain histological studies (e.g., Clémencon, 2012; Reijnders, 1983) described only faint and loose veil-like hyphae. Finally, to distinguish cyphelloid fungi from state 0, we produced a five-character state coding (5ST).

To examine the effects of multiple binary traits in one model, we created a binary coding by merging the semi-enclosed with the enclosed state of the 3ST coding regime into one state (2ST1). This appeared feasible because these two states behaved similarly in the trait dependent diversification analyses. In addition to this, we created a binary coding where semi-enclosed and open development were merged (2ST2) and where we randomly distributed the semi-enclosed state between species with enclosed or open development states (2ST3).

2.1.2. Partial and universal veil and hymenophore

We coded partial and universal veil character states (Fig. 1) for each of the species in the phylogeny as two binary traits by following the definitions in (Clémencon, 2012) (Supplementary Data 1, Supplementary Methods). In a few cases, the nature of a veil was hard to define, therefore we created a veil coding where species with any of the veils were coded as state 1 and species without veils to state 0.

We included two additional traits that could influence diversification: cap formation and increased hymenophore surface area. We obtained character coding for the cap from Varga et al., 2019. For the hymenophore, we distinguished three character states based on the structural complexity of the hymenophore (Fig. 2, Supplementary Data 1). State 0 and 1 was assigned to species with a smooth hymenophore and weakly-structured hymenophore, respectively. Weakly-structured hymenophore, such as those comprised of veins, ridges or bumps barely increases the hymenophore's surface. State 2 was assigned to species with complex hymenophores (e.g., gills, pores, teeth) (Fig. 2) which remarkably expands the spore-bearing surface. For the multitrait binary model, we merged the smooth and weakly-structured states into state 0, and we assigned state 1 to species with complex hymenophore.

2.2. Character state evolution

First, for all the above-mentioned traits, we wanted to test if evolution has a tendency towards a state by inferring character state transition rates and performing model tests. All character state evolution analyses were performed on 245 Maximum likelihood (ML) phylograms or ten chronograms obtained from previous work (Varga et al., 2019), depending on the evolutionary model used. For a detailed description of tree-inference see Supplementary Methods and the methods in Varga et al. (2019). To infer macro-evolutionary transition rates, we used ML and Markov Chain Monte Carlo (MCMC) approaches implemented in BayesTraits 2.0 Linux 64 Quad Precision alternative build (Meade and Pagel, 2016) and in diversitree 0.9–10 R (Fitzjohn, 2012). BayesTraits analyses were performed on 245 phylogenies using the Multistate module of
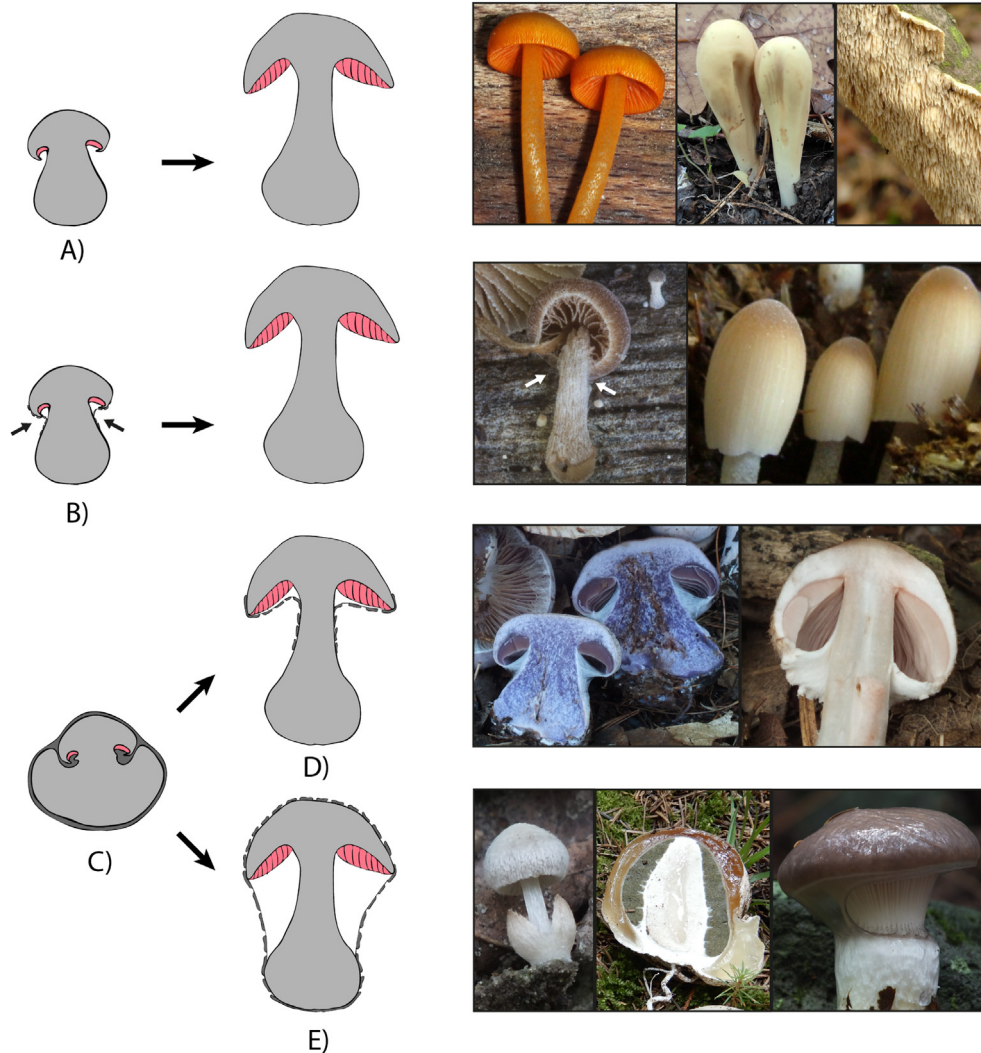
Fig. 1. Developmental types in mushroom-forming fungi. Drawings depict primordial (left) and young fruiting bodies (right) of different developmental types. Magenta color shows the hymenial tissues. Dark grey color indicates tissues having role in the enclosure of the developing fruiting body A) Open development state. Images (left to right): Mycena leiana, *Clavariadelphus pistillaris* and *Irpex lacteus* B) Semi-enclosed development state. Note the faint tissue layer covering the hymenium of the primordium (arrowheads). Images (left to right): Ramicola sp. and *Coprinellus congregatus*. C) Enclosed development state; a robust tissue covers either the whole primordium or the hymenophore. D) and E) are subtypes of the enclosed development state showing partial and universal veils, respectively. D) Young fruiting body with partial veil Images (left to right): Cortinarius sp., Agaricus silvaticus. E) Young fruiting body with universal veil. Images (left to right): Volvariella sp., *Phallus impudicus. Gomphidius glutinosus*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the program, under a gamma-distributed hyper-prior for transition rates (Supplementary Data 2), which empirically fit best the data based on preliminary analyses with uniform, exponential and gamma priors with and without a hyper-prior.

All preliminary BayesTraits analyses (prior selection, constraining deep nodes, clade specific character state coding) were conducted with the following settings: 1,010,000 generations, 10,000 burn-in and sampling every 500th generation. We observed that MCMC generally visited only ~15 out of the 245 trees, which means 230 trees did not contribute toward our results. To overcome this in the analysis of the 3ST regime of the developmental types, the partial and universal veil traits, and the hymenophore complexity trait, we forced the Markov chain to spend 200,000 generations on each tree by the *EqualTrees* command and set burn-in to 100,000 and sampling every 500th generation (altogether 49 million generations). In the case of 4ST1, 4ST2, and 4ST3 coding regimes, Markov chains were run for 10 million, while in the case of 5ST for 20 million generations with 10% burn-in and sampling every 500th generation.

Marginal likelihoods were estimated during the analysis of the 3ST coding regime by the stepping stone method (Meade and Pagel, 2016; Xie et al., 2011) using 50 stones with chain lengths of 5000. Analyses were repeated three times to check congruence of independent runs. We performed model tests by comparing the unconstrained model and a nested model where certain constraints were made on the parameters. First, we tested if there is a tendency towards the evolution of any character states by constraining forward and reverse transition rates to be equal. To explore if a particular transition rate is supported by the data, we set the rate to zero ($q_{10} = 0$ or $q_{01} = 0$ or $q_{21} = 0$ or $q_{12} = 0$ or $q_{20} = 0$ or $q_{02} = 0$). Each of the constrained models mentioned above were compared to the best fit model using log-likelihood ratios (LR, ML analyses) or the log marginal likelihood ratio (Bayes factor (BF), MCMC analyses). As a rule of thumb LR > 4 or BF > 10 was considered as significant support (Pagel, 1999). We also compared the above-mentioned models under the multistate speciation and extinction (MuSSE, Fitzjohn, 2012) or the binary state speciation and extinction (BiSSE, Maddison et al., 2007) models for the enclosed development 3ST
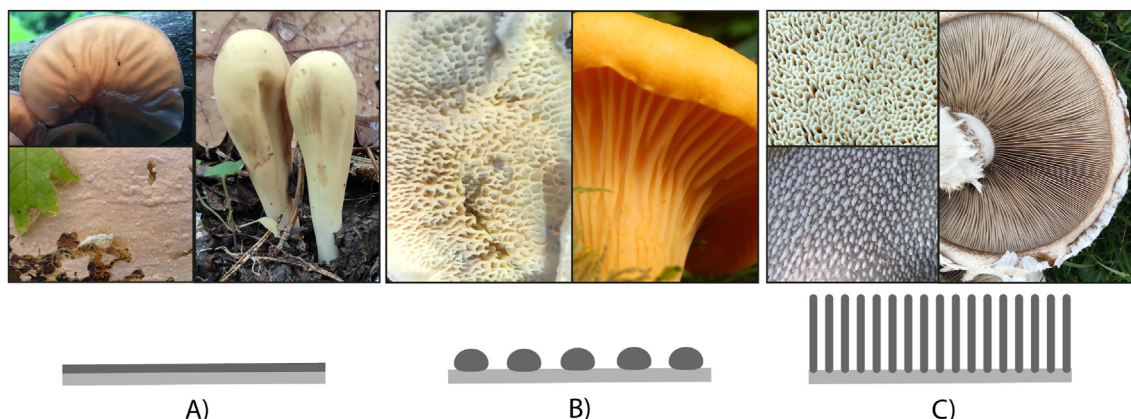
Fig. 2. Three states/grades of hymenophore complexity distinguished in this study. A) Smooth hymenophore. Images: *Auricularia auricula*-judae (top), *Clavariadelphus pistillaris* (right) and Cylindrobasidium sp (bottom). B) Weakly-structured hymenophore. Images: *Phlebia tremellosa* and *Cantharellus cibarius*. C) Complex hymenophore. Images: *Bondarzewia montana* (top), Nemecomyces mongolicus (right) *Hydnum repandum* (bottom). In schematic figures grey and dark grey denote supporting tissue (e.g., trama, subiculum) and sporogenous tissue (hymenium), respectively.

and 2ST1, hymenophore, universal veil and partial veil traits. For detailed parameters see the following paragraphs.

Because our main hypothesis was that enclosed development affects the evolution of mushroom-forming fungi we performed a more thorough analysis of this trait than others. Previous studies showed that the resupinate morphology, which is an open development type, is the ancestral state in Agaricomycetes (Hibbett and Binder, 2002; Sánchez-García et al., 2020; Varga et al., 2019). However, we observed high rates of transition from semi-enclosed to open development, which we hypothesized was caused by a spurious early gain of semi-enclosed, followed by frequent reversals to open development. To address this, we performed two additional tests. First, we examined whether constraining the stem nodes of 13 class- or order-level clades (Supplementary Fig. 1) to open development affects the transition rates. Second, we examined the contribution of individual clades (Supplementary Data 1 and 3) to transition rates by setting state 1 or 01 of all species in a clade at a time to 0 and state 12 to 2. The rationale of this test was that a dramatic change in the transition rates relative to the original values could mean the given clade is a main contributor to the global pattern.

2.3. Ancestral state reconstruction

By inferring transition rates (see above) we did not get information about ancestral character states. Therefore, we inferred the ancestral states in of developmental types, the hymenophore complexity, partial and universal veil traits, and the presence/absence of the cap (the latter trait was published in Varga et al., 2019). To find the joint ML estimates of ancestral states given one of the chronograms we used the *corHMM* function of the corHMM v.2.7 R package (Beaulieu et al., 2021) under an "all rates differ" model and 100 random starting points during the ML search. Using a custom R function (see Data Availability) and the results of the corHMM function, we calculated the number of origins of each state by counting the node-wise transitions between states given the joint estimates of ancestral states. We also calculated the number of state transitions by excluding tip states from the calculations to rule out the effect of ambiguous character state coding affecting individual species.

2.4. Trait-dependent diversification analyses

2.4.1. BiSSE and MuSSE models

Next, we fit models that can describe not just the directionality of evolution between the character states but the effect of character states on the speciation and extinction rates of species. We used ten chronograms from previous work (Varga et al., 2019) to analyze state-dependent diversification using the MuSSE or the BiSSE models implemented in diversitree v.09—10 R (Fitzjohn, 2012). The ten chronograms were randomly selected to cover the tree space of the 245 phylogenies (for more details see Varga et al., 2019 and Supplementary Methods). Transition, speciation, and extinction rates were inferred by using both ML and Bayesian MCMC. Starting points of ML searches were determined by the functions *starting.point.musse, starting.point.bisse,* and the analyses were corrected by state-specific sampling fractions (Supplementary Data 4) calculated by using the procedure of Varga et al. (2019). Bayesian MCMC was performed using an exponential prior for all parameters (calculated by the make.prior.exponential function and using a rate parameter defined as *1/(2r),* where *r* is the character independent diversification rate given by the starting.point.bisse or starting.point.musse functions) and Markov chains were run for 20,000 generations with 10% burn-in. The MCMC sampler's step size was optimized after running 100 generations. Convergence was assessed based on the variation of parameter values as a function of the number of generations.

Differences among alternative models were determined by Akaike information criterion scores, and by likelihood ratio test (LRT) where $P < 0.05$ was considered to be significant. We constrained state-specific speciation or extinction rates to be equal ($\lambda_0 = \lambda_1$ or $\lambda_0 = \lambda_2$ or $\lambda_1 = \lambda_2$ or $\mu_0 = \mu_1$ or $\mu_0 = \mu_2$ or $\mu_1 = \mu_2$) and performed LRT. We also tested whether a particular speciation or extinction rate is a significant component of the model by constraining it to zero ($\lambda_0 = 0$ or $\lambda_1 = 0$ or $\lambda_2 = 0$ or $\mu_0 = 0$ or $\mu_1 = 0$ or $\mu_2 = 0$).

In the case of the developmental types 2ST3 coding regime, we generated 100 perturbed traits by randomly distributing the semi-enclosed state between the two other states. Using this dataset and ten chronograms we performed 1000 ML BiSSE analyses to infer transition, speciation and extinction rates. By inspecting the distribution of the 1000 ML parameter estimates we wanted to find out whether reassigning states to species harboring semi-enclosed development could affect the observed pattern of diversification rates.

2.4.2. Multitrait MuSSE model

To analyze the effect of multiple binary traits on speciation and extinction rates in one model, we used the multitrait MuSSE model (Fitzjohn, 2012). The parameterization of the multitrait MuSSE model is analogous to that of a linear regression model.

Consequently, an intercept (a "background rate") and main effects (the effect of state 1 of any traits) are inferred. We analyzed a multitrait MuSSE model with the enclosed development, cap and hymenophore traits included. First, by performing ML analyses using a sampling fraction 0.149, we compared the model where only the intercept was inferred ("depth" argument = c(0,0,0)) with the model where the main effect of speciation and extinction were included ("depth" argument = c(1,1,0)) by using LRT. We also examined the significance of the main effects by performing LRT on models where the effect of one of the traits was constrained to be 0 ($\lambda_A = 0$ or $\lambda_B = 0$). Finally, we performed Bayesian analysis by generating 40,000 generations from which the first 10,000 generations were discarded as burn-in. We compared the posterior distribution of parameter estimates of the multitrait MuSSE model and that of BiSSE models to examine how the speciation and extinction rates changed when the three traits were analyzed within one model. In addition to the above-mentioned analyses, we inferred the parameters of a model where the effect of the partial veil and universal veil and their interactions were included ("depth" argument = c(2,2,1)). With this ML inference, we wanted to test if the effect of the two veil structures is additive given the defined model.

2.4.3. Hidden state speciation and extinction (HiSSE) model

To rule out the possibility that the diversification rate pattern of developmental types is driven by an unobserved trait, we performed analyses under the HiSSE model (Beaulieu & O'Meara, 2016) implemented in the *hisse* R package v.1.9.19. Following the procedure of Beaulieu and O'Meara (2016) we inferred parameters of five models. First, a character-independent diversification model (CID) where only one speciation and extinction rate were included independently from any trait. Second and third we performed analyses using the BiSSE model and a HiSSE CID-2 model, respectively. The HiSSE CID-2 model has the same number of parameters as the BiSSE model but these account for the evolution of an unobserved trait. Fourth, we built a model where an observed binary trait (developmental types or cap or hymenophore complexity) and an unobserved trait were included (HiSSE full model). Finally, we analyzed a model with the same complexity as the HiSSE full model, but both traits were unobserved, hidden traits (CID-4). We calculated the Akaike weights among the five models to find out which model fit best to our data. Next, to explore the uncertainty in model parameter estimates we performed a Bayesian analysis on a HiSSE full model using the RevBayes v.1.1.0 program (Höhna et al., 2016). Following the RevBayes manual (Höhna et al., 2019), we set up model priors (see also Supplementary Methods) and we ran three independent chains for 4500 generations each. Convergence was assessed based on saturation of parameter estimates. We discarded samples prior to convergence and calculated effective sample sizes (ESS) for all parameters by the *effectiveSize* function in the coda v.0.19—3 package (Plummer et al., 2006).

3. Results and discussion

3.1. Enclosed development is the favored direction of evolution

Innovations in reproductive ability influence the evolutionary success of species (Heard and Hauser, 1995); consequently various solutions exist to protect developing youth, including nursing and protective mechanisms (Blackburn, 1999; Roberts et al., 2016). Animals evolved strategies for parental care, such as viviparity that increased the net diversification rate of the lineages (Helmstetter et al., 2016; Pyron and Burbrink, 2014), however, how general these are among other organisms is not known. In fungi, spore-producing cells are lifted aboveground to increase spore dispersal

efficiency, however, this exposes these cells to environmental impacts. Many strategies evolved to protect spore-producing cells (e.g., Braga et al., 2015); among these the ensheathment of the spore-producing cells into a protected environment (enclosed development), is a widespread mechanism. However, how its evolution impacted species' success (i.e., speciation rate) is not known.

Here, we first examined whether there is a trend in the evolution of developmental types in mushroom-forming fungi (Agaricomycetes) using comparative phylogenetic methods on previously published phylogenies of 5284 species (Varga et al., 2019). We found significant asymmetries in transition rates among developmental types based on the ML and Bayesian analyses under the MuSSE model using 10 chronograms (Fig. 3). The highest average transition rates were inferred for the transition from the semi-enclosed to the enclosed state (q_{12}); these rates were 8.6—21.0 times higher than the reverse rates (q_{21}). Our results also suggest that the reversal from semi-enclosed to open development (q_{10}) is frequent across the phylogeny. Model comparisons indicated that these asymmetries were significant in all cases (LRT, P < 0.05, log BF > 10; Supplementary Data 4 and 5) and that all transition rates were crucial parameters of the model (i.e., significantly greater than zero, LRT, P < 0.05, BF > 10). These results were also supported by parameter inferences and model tests implemented in BayesTraits using mk models (Supplementary Note 1 and Supplementary Data 2).

ML based ancestral state reconstructions suggest that open, semi-enclosed and enclosed development evolved 93—115, 78—104 and 123—172 times respectively. We found a high effect of terminal branches on the number of state transitions (Supplementary Data 6). We suspected this was caused by uncertainties in character state coding, however, we found that our results are robust to alternative character coding regimes and other potential sources of subjectivity we identified (Supplementary Note 1).

Overall, these analyses suggest that enclosed development is a frequently-evolving and stable character state and its evolution is the preferred direction in mushroom-forming fungi. It may emerge either via a semi-enclosed intermediate (mean rate, $q_{01} = 0.02$ and $q_{12} = 1.16$) or could directly evolve from open development (mean rate, $q_{02} = 0.10$). Given that this developmental type provides the strongest physical protection from the environment of the three character states (but also requires the largest nutritional investment), it is conceivable that it confers a fitness advantage for mushroom-forming fungi, especially for those that produce above-ground fruiting bodies. On the other hand, the semi-enclosed state appears evolutionarily labile; once evolved, it either transforms into the enclosed state or is lost rapidly (reversal to open), possibly due to the fugacious, incomplete protection it can provide to fruiting body initials. Convergent evolution of closed forms was also speculated in the Pezizomycotina (Ascomycota), where species with open fruiting bodies (apothecia) were basal to those with closed ones (perithecia) (Liu and Hall, 2004). In lichen-forming ascomycetes (Lecanoromycetes) several independent transitions to closed (angiocarp) fruiting bodies were detected (Schmitt et al., 2009).

3.2. Enclosed development is associated with an elevated species diversification rate

We evaluated the impact of enclosed development on speciation and extinction rates using state-dependent speciation and extinction (SSE) models. Species with semi-enclosed development have the highest net diversification rate (range of mean values across analyses: $6.5 \times 10^{-2} - 8 \times 10^{-2}$ events per million y), followed by species with enclosed ($5.5 \times 10^{-2} - 6.1 \times 10^{-2}$) and open
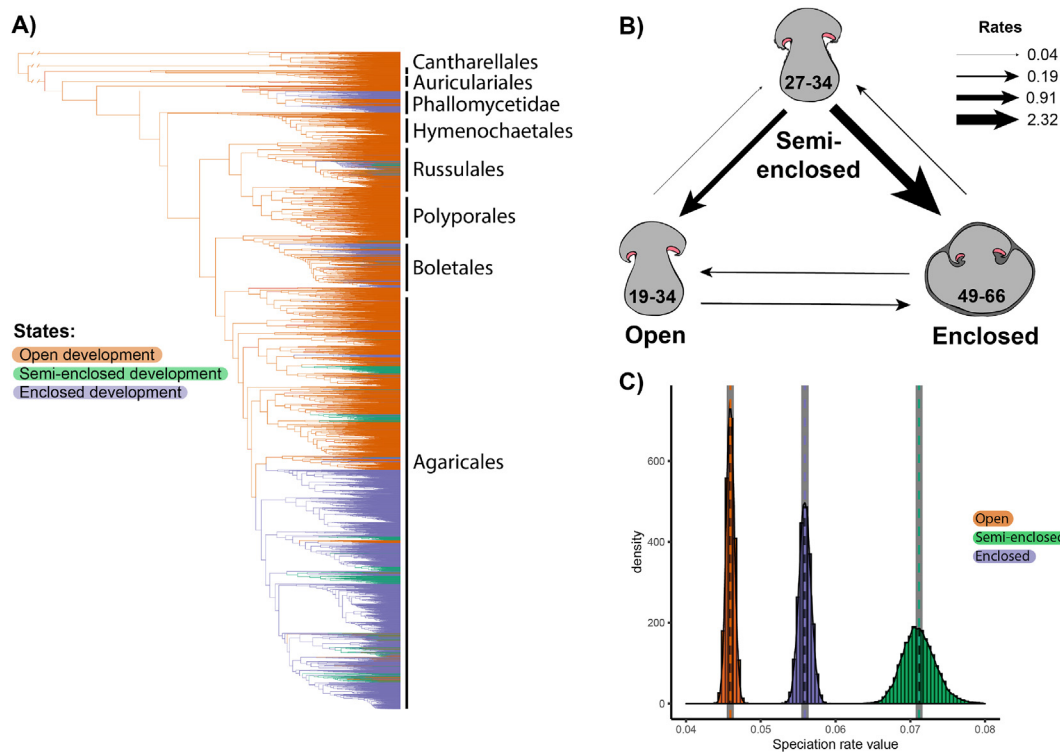
Fig. 3. Macro-evolutionary patterns of enclosed development. A) Maximum parsimony ancestral state reconstruction of the 3ST coding regime. State 0 — Open development, State 1 - Semi-enclosed development, State 2 — Enclosed development. B) Evolutionary transitions between state 0, state 1 and state 2. Number intervals on each schematic graphics of the states show the number of times a state evolved as inferred by maximum likelihood. Arrows denote transition rates between states, their width is proportional to the mean transition rates inferred by BayesTraits. C) Histograms show the posterior probability distribution of state-dependent speciation rates inferred by MuSSE.

development ($4.6 \times 10^{-2} - 5.2 \times 10^{-2}$) (Fig. 3, Supplementary Data 4), based on analyses of ten chronograms under the MuSSE model using ML or Bayesian methods. We found that speciation rate drove the differences in net diversification rates, because 26 out of 30 model tests showed significant differences in speciation rates (LRT, $P < 0.05$), but non-significant differences between any pair of extinction rates (Supplementary Data 5). These results were robust to merging the semi-enclosed character state with either of the other two states and to randomly distributing semi-enclosed species among other states (essentially reducing it to a BiSSE) (Supplementary Note 1).

The most species-rich clade (3387 species) where 50% of the taxa produce a fruiting body with enclosed development is the Agaricoid clade *sensu* Matheny et al. (2006) in the Agaricales, consisting of enigmatic genera such as *Agaricus*, *Cortinarius* or *Laccaria*. Outside Agaricales the Phallomycetidae subclass (e.g., *Phallus* spp., *Geastrum* spp., *Hysterangium* spp.), Russulales (e.g., certain *Lactarius* spp., *Gymnomyces* spp.), and the Boletales (e.g., *Scleroderma* spp., *Rhizopogon* spp., *Astreus* spp.) consist of many species with enclosed development.

We hypothesize that the elevated diversification rate stems from improved adaptation to terrestrial habitats conferred by the protection of fruiting body development, regardless of the complexity or the persistence of the given structure. In concordance with this, evidence from molecular and gene expression studies also supports the protective role of fruiting bodies against environmental impacts. It has been shown that secondary metabolites, peptides and proteins (e.g., galectins) against bacteria or fungivorous animals (mammals, arthropods, nematodes) are produced by tissues that ensheath fruiting body initials (Bleuler-Martínez et al., 2011; Boulianne et al., 2000; Jaeger and Spiteller, 2010; Künzler, 2018; Sabotič et al., 2011, 2016). Enclosed

development shares features with internally nursed embryos of viviparous animals and plants, though it is important to note that similarity is limited to the internal protection of vulnerable stages of development, as the fruiting body initial serves a different purpose from the plant/animal embryo. In analogy, nevertheless, viviparity spurred lineage diversification in squamates and cyprinodontiform fishes (Helmstetter et al., 2016; Pyron and Burbrink, 2014), further suggesting that the protection of vulnerable structures can contribute to the evolutionary success of species.

3.3. Both partial and universal veils contribute to the diversification rate increase

Protection of fruiting body initials in species with enclosed development is provided by at least two morphological structures, partial and universal veils, both of which could potentially drive increased diversification rates we inferred above. The partial veil covers the hymenophore (gills, pores) by stretching between the stem and the edge of the cap, whereas the universal veil envelopes the whole fruiting body when young. As the majority of species with enclosed or semi-enclosed development possess at least one kind of veil (Fig. 4), we attempted to dissect their individual contributions to diversification rate differences. The net diversification rate of species with universal or partial veils, respectively, was 1.23 and 1.33 times higher than that of species without either veil type (Supplementary Fig. 2). As in the case of developmental types, diversification rate differences appear to be driven by differences in speciation rate, not extinction rate (LRT, $P < 0.05$). We found similar results when the universal and partial veil traits were combined into one trait (Supplementary Data 4 and 5). We also analyzed a multitrait MuSSE model where the simultaneous effects of the partial and universal veils and their interaction were defined and
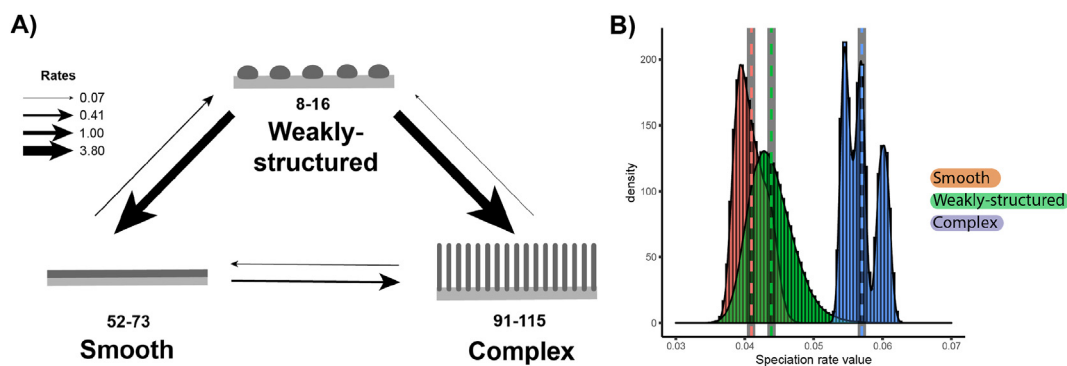
Fig. 4. Macro-evolutionary patterns of hymenophore complexity (see also Fig. 2 for organismal examples). A) Transition rates between the three character states (smooth hymenophore, weakly-structured hymenophore, complex hymenophore), inferred by BayesTraits. The intervals below the schematic graphics represent the number of times a state evolved according to maximum likelihood ancestral state reconstruction. The width of the arrows is proportional to the transition rates. B) Histograms depicting the state dependent diversification rates of the three character states inferred by MuSSE.

found that the effect of partial and universal veils is not additive given our data (Supplementary Fig. 3). Despite this, the results suggest that both universal and partial veils contribute to increased diversification rates in species with (semi-) enclosed development. Although the exact ways in which veils increase fitness remain unknown at the moment, the upregulation of insecticidal and nematocidal toxin producing genes in veil cells (Boulianne et al., 2000; Sabotič et al., 2011) suggests that veils may form both chemical and physical barriers. Enclosed development might thus help phasing the growth of fruiting bodies, by providing a sheltered environment for cell differentiation, after which rapid growth by cell expansion (Kües, 2000; Nagy et al., 2022) lifts spore producing cells in the hymenophore quickly above ground. This might be advantageous in terrestrial habitats, where developing at ground level and lifting the cap above ground reduces evaporation and potentially allows the development of larger fruiting bodies, which increase spore quantity and release height, two critical factors in dispersal (Norros et al., 2014).

3.4. Multiple traits drive mushroom diversification

Observed morphological traits. Diversification rate differences can be driven by single traits or by interactions between multiple traits (Rabosky and Goldberg, 2015). We therefore tested whether the observed impact of developmental type on diversification rate could have been influenced by phylogenetically co-distributed characters.

Multiple studies have suggested that complex hymenophores (e.g., gills, pores) increase the spore-to-biomass ratio (Clémencon, 2012; Fischer and Money, 2010). Therefore, we hypothesized that lineages with complex hymenophores have higher net diversification rates than those with simple hymenophores. Based on MuSSE and BayesTraits we found asymmetries in the transition rate patterns of hymenophore morphologies (Fig. 4., Supplementary Note 1). We also found that the diversification rate of species with complex hymenophore was significantly higher than that of species with smooth or weakly-structured hymenophore (LRT, P < 0.05), while diversification rates of species with the latter two did not differ significantly (Fig. 5, LRT, P > 0.05, Supplementary Data 2 and 5). These results suggest that only well-developed gills, pores, and teeth can positively affect diversification of mushroom-forming fungi, whereas weakly-structured hymenophores (bumps, ridges, veins) do not and they revert frequently to smooth hymenophore surface. Increased net diversification of species with complex hymenophore could be related to the production of more propagules per unit biomass (Iapichino et al., 2021), variations in gill

positioning (Fischer and Money, 2010), protection against predators (Nakamori and Suzuki, 2007), keeping high humidity (Halbwachs and Bässler, 2015) or producing local winds that help spores dispersal (Dressaire et al., 2016).

Next, we examined the simultaneous effect of developmental type, morphological complexity of the hymenophore and the presence of a cap in a multitrait speciation and extinction model (multitrait MuSSE). The presence of a cap has been shown to have positive effect on net diversification rate (Varga et al., 2019) and it is a widespread trait (Fig. 5). The multitrait MuSSE model allowed us to decipher the background and the individual ("main trait") effects of binary traits on diversification rate, and thus to separately evaluate the contribution of each trait to diversification rate changes (Fitzjohn, 2012). In the multitrait MuSSE framework the models with main trait effects were superior over the model with only the "background" effect (LRT, P < 0.05). We found that developmental types, the hymenophore and the cap were all significant components of the model with main trait effects, because the log-likelihoods of the unconstrained models were significantly higher than that of models where the effect of one or the other trait was constrained to zero (LRT, p < 0.05). We found that the speciation rate differences under two states (λ_1-λ_0, called 'trait effect') in the multitrait analyses were lower than those in the BiSSE analyses (Fig. 4). This indicates that speciation rate differences inferred under BiSSE are, to an extent, arise from the interaction of three traits. However, the trait effects remained significantly higher than zero in all cases (λ_1-λ_0>0, LRT, P < 0.05, Fig. 5) indicating a robust and independent positive impact on diversification by each of the three states (enclosed development, complex hymenophore, or the presence of a cap). This suggests that the increased speciation of species with enclosed development is robust to inclusion of both the complexity of the hymenophore or the presence of a cap traits.

Effects of hidden states. To address the possibility that unobserved or hidden traits drove the observed patterns, we performed a hidden state speciation and extinction (HiSSE) analysis. This model includes one or more hidden traits to test if a trait not considered by the research is responsible for the observed diversification pattern. We found that the model where an observed (developmental types or cap or hymenophore) and a hidden trait were included (HiSSE full model) was supported over the BiSSE or the three different CID models (Supplementary Table 1). This suggests that the diversification rates were not driven by solely one trait but by multiple traits including our focal observed traits. We also measured the variance of the parameter estimates of the model with enclosed development as an observed trait using Bayesian analysis. We found that the speciation rate of species with
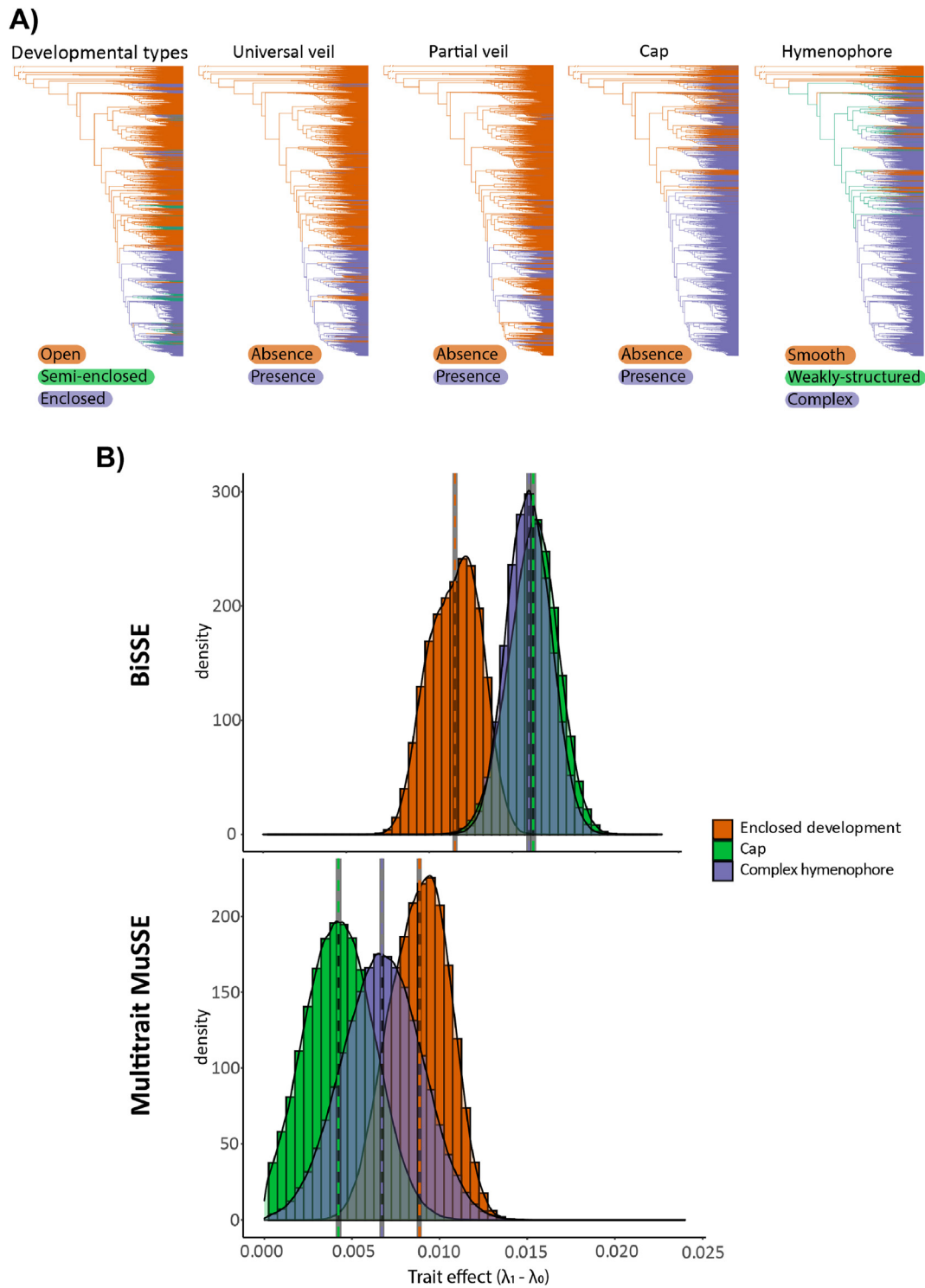
Fig. 5. A) Maximum likelihood ancestral state reconstruction of five morphological characters examined in this study. Developmental type (open development, semi-enclosed development, enclosed development). Universal veil, partial veil and cap trait (absence, presence). Hymenophore complexity trait (smooth hymenophore, weakly-structured hymenophore, complex hymenophore). B) speciation rate effects (λ_1-λ_0) inferred by BiSSE (upper row) and multitrait MuSSE (bottom row) analyses.

enclosed development was higher than that of species with non-enclosed development (Supplementary Fig. 4, Supplementary Data 4), even in the presence of a hidden trait, suggesting that the diversification rate patterns we identified are indeed attributable to innovations in developmental mode.

4. Conclusions

The evolutionary success of species is, among others, connected to their reproductive efficiency. Accordingly, the impact of innovations on reproductive ability influence which morphologies,

behaviors or other traits reach high equilibrium frequencies or go extinct in their clades. In the context of fungi, traits related to spore production, dispersal and germination are among the primary determinants of reproductive success in terrestrial habitats (Aguilar-Trigueros et al., 2019; Halbwachs et al., 2015; Hibbett and Binder, 2002; James, 2015; Norros et al., 2014; Peay et al., 2012). In this respect, fruiting bodies offer tremendous advantages in fungi by protecting developing spores, lifting them above ground and facilitating their dispersal. Selection of fruiting body traits should, thus, drive morphological evolution and may impact lineage diversification. Despite this clear prediction, what adaptations fruiting bodies evolved for increasing spore dispersal efficiency are hardly known and studies addressing the correlations between morphogenetic traits and species diversification are at paucity.

So far, the effect of the main fruiting body types (Sánchez-García et al., 2020), the pileate (Varga et al., 2019), the gasteroid morphology (Wilson et al., 2011), main nutritional modes (Sánchez-García et al., 2020) and ectomycorrhizal lifestyle (Sánchez-Garcia et al., 2017) on the speciation rate has been examined among mushroom-forming fungi. It has been shown that the major drivers of increased speciation rate of species are morphological innovations (Sánchez-García et al., 2020) and that ecological innovations such as the evolution of the ectomycorrhizal lifestyle (Matheny and Fordyce, 2019; Sato and Toju, 2019) had a minor impact on the diversification of mushroom-forming fungi.

In this study, we provide evidence that morphological innovations pertaining to the efficiency of spore production show considerable asymmetry in their gain/loss rates and their emergence is associated with increased net diversification rates (i.e., may be key innovations) in mushroom-forming fungi. These include enclosed development, in which fruiting body initials develop internally and are protected by various outer tissue layers. these layers (veils, peridium) provide protection to the fruiting body initial, in a manner analogous to the internally nursed embryos of viviparous animals and plants (Helmstetter et al., 2016; Pyron and Burbrink, 2014). Moreover, we showed that the two main protective tissues, universal and partial veils, contribute to enclosed development among mushroom forming fungi provide alone or in combination an elevated net diversification to lineages. The increased speciation rate of lineages with enclosed development could stem from the ability of protecting fruiting body initials and the developing basidia and spores therein. Several factors can compromise development, such as desiccation, predators, infections, rain, other physical damages, and consequently impede sporulation. Enclosed development could help fending off these challenges and thus might allow fungi to invade terrestrial niches, such as dry above-ground sites, that are not available to species in which fruiting body initials are exposed during early development.

At large evolutionary scales such as the one examined in this paper, causes of diversification rate differences may easily be distributed among a nested set of phenotypic innovations or phylogenetically co-distributed traits (Donoghue, 2005). To address this possibility, we first examined the effects of additional two independent, but conceivably adaptive traits, the presence of a cap and structured hymenophore surfaces in integrated analyses. Multitrait MuSSE models provided evidence that, albeit both traits influence diversification rates (see also Varga et al., 2019), the positive effect of enclosed development on species' speciation still persists. Finally, by using a hidden state speciation and extinction model (Beaulieu & O'Meara, 2016), we showed that developmental type was a significant component of the model, and species with enclosed development exhibited increased net diversification, yet, further unobserved traits could also have influenced the speciation rate of mushroom-forming fungi.

Overall, this study identified new traits that influence lineage diversification in mushroom-forming fungi and facilitates the functional and evolutionary interpretation of complex morphological innovations in one of the best-known fungal groups. Our results suggest that the extant diversity of Agaricomycetes has probably been influenced by a complex interplay between multiple fruiting body and, possibly also nutritional innovations and that phylogenetically nested sets of these may underlie the radiation and evolutionary success of mushroom-forming fungi, one of the most diverse, important and spectacular components of the terrestrial ecosystems.

5. Data availability

Phylogenetic input data from Varga et al., (2019) can be accessible at Dryad (accession number: https://doi.org/10.5061/dryad.gc2k9r). The custom R function used to calculate the number of origins based on ancestral state reconstruction is available at github.com/vtorda/ASR_analysis. All other datasets can be found as part of the supplementary material.

Author contributions

L.G.N and T.V. designed the study and wrote the manuscript. Cs.F. analyzed the hymenophore trait, all other analyses were performed by T.V. All authors contributed to morphological data collecting and revising the manuscript.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2022.07.001.

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