Examining the macro-evolution and genetic background of complex multicellular structures in mushroom-forming fungi (Agaricomycetes)

Theses of doctoral (Ph.D.) dissertation

Varga Torda

Supervisor: Dr. László G. Nagy

Synthetic and Systems Biology Unit, Institute of Biochemistry, Biological Research Centre

Doctoral School of Biology, Faculty of Science and Informatics, University of Szeged

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Introduction

Multicellular organisms exist in all biogeographic realms and are essential components of most present ecosystems. Simple multicellular organisms form aggregates or colonies of cells and have arisen in both prokaryotic and eukaryotic lineages at least 25 times. In contrast, complex multicellularity, where three-dimensional structures are developed and only some of their cells are in direct contact with the environment, evolved exclusively in five eukaryotic lineages from which three dominate the present terrestrial ecosystems: animals, embryophytic land plants and fungi. Despite the prevalence of complex multicellularity on Earth, the driving force and the genetic background of the evolution of complex multicellular organisms are incompletely known. The fungal kingdom is a unique complex multicellular lineage because complex multicellularity could have convergently appeared at least eight times within fungi. Possibly, the most common fungal complex multicellular structures are the sexual fruiting bodies, whose primary purpose is to produce meiotic spores in a protective environment and facilitate spore dispersal.

Agaricomycetes, also called mushroom-forming fungi, contain more than 20,000 species, which produce fruiting bodies. Therefore, examining this class of fungi with phylogenetic

comparative methods (PCMs) would widen our knowledge on the macro-evolution of complex morphologies in fungi.

Despite the efforts of several researcher in the past years the genetic background of mushroom development is still poorly known. One of the best-examined mushrooms is the *Coprinopsis cinerea* model organism. *C. cinerea* is a saprotrophic fungus, easy to grow under laboratory conditions and have fast (1-2 week) life cycle. The genome of the *C. cinerea* was sequenced a decade ago and since then, numerous knowledge has been gathered about this mushroom. However, it is still obscure, how the main tissue types start to develop and what are the genes which have a role at the beginning of the development of *C. cinerea*.

Objectives

We aimed to investigate the macro-evolution of the largest class in the fungal kingdom, the mushroom-forming fungi (Agaricomycetes), which contains complex multicellular structures. We wanted to examine the evolution of fruiting bodies and related structures such as the cap, protective sheaths and spore-bearing structures (hymenophores). We further wanted to disentangle the genes which play a role in the formation of the fruiting body and its main tissue types. Therefore, we set up an experiment using a low-input RNA-seq method coupled with laser-capture microdissection and examined the transcriptome of seven stages and nine tissue types through the early development of the gilled model mushroom, *Coprinopsis cinerea*. More specifically, we attempted to answer the following questions:

1. What was the tempo of the speciation and extinction of species through the evolution of mushroom-forming fungi?
2. Was there any rapid rate shift regarding speciation or extinction rates indicating key innovation or mass extinction events, respectively?
3. If yes, could it relate to morphological traits?

4. Could morphological traits increase the diversification rate of species? We asked this question regarding six traits: cap production, the increased surface area of hymenophore, enclosed development, presence of protecting sheaths like universal veil or partial veil.

5. What genes are expressed during the transition from vegetative mycelium to primary and secondary hyphal knot developmental stages of *Coprinopsis cinerea*?

6. Could hyphal growth pattern changes be recognized at the transcriptome level of fruiting body development?

7. Are defense and surface coating associated genes more characteristic for cap, universal and partial veil than for other tissues?

8. What are the tissue-enhanced genes and when did them appear during evolution?

Methods used

- Maximum likelihood (ML) phylogenetic tree inference (RAxML), Two-step Bayesian molecular clock calibration of phylogenetic trees (PhyloBayes, FastDate). Bayesian or rate smoothing molecular clock calibration of phylogenomic data (mcmctree and r8s, respectively).
- Modeling discrete trait evolution: Mk models implemented in BayesTraits program.
- Parsimony and stochastic character mapping based ancestral state reconstruction analyses.
- Trait independent diversification rate analyses (BAMM)
- Trait dependent diversification analyses (BiSSE and MuSSE)
- Producing tissue-specific RNA-seq libraries with the use of
    - Cryo-histological methods
    - Laser-capture microdissection
    - Low-input RNA-seq methods
- Read alignment and quality check: STAR RNA-seq aligner, FastQC program, BBMap package, EDAseq package.
- Expression analyses with negative binomial (NB) generalized linear model (GLM): DESeq2 package, independent hypothesis

weighting (IHW), GO, IPR, KOG enrichment analyses using topGO package and hypergeometric test.

- Genomic phylostratigraphic analysis

Summary of the results

Producing robust phylogenetic trees and chronograms of the class Agaricomycetes

We gathered specimens of Agaricomycetes from every geographical region except Antarctica and assembled a dataset containing 5,284 species and three genomic loci (nrLSU, rpb2, tef1-α). Using this dataset, we performed maximum likelihood (ML) inference, and we constrained the topology of the backbone based on a phylogenomic tree consist of 105 species. Overall, 245 ML trees were inferred, covering the topological variety of Agaricomycetes phylogeny based on Robinson-Foulds pairwise distances. We also performed molecular clock calibration on ten trees selected by stratified random sampling to represent the topology diversity of the 245 trees. The molecular clock calibration was done by a two-step time calibration strategy resulting in ten chronograms.

The diversification of Agaricomycetes lineages abruptly increased in the Jurassic-period

We inferred branch-specific speciation and extinction rates and summarized the net diversification rates (speciation minus extinction rates) over time. We found that the diversification rate abruptly increased in the Jurassic period (200-145 Ma ago).

Agaricomycetes was affected differently by mass extinction events than other organisms

A small increase in the net extinction rate was also detected at the end of the Jurassic period. Thus, we further scrutinized whether mass extinction could have occurred through the evolution of Agaricomycetes using the CoMET model. This analysis also supported the presence of an extinction event at the end of the Jurassic period, but interestingly, no sign for a mass extinction at the Cretaceous-Paleogene (K-Pg) boundary was detected. Therefore, mushroom-forming fungi probably did not suffer severe extinction at the K-Pg boundary as most of the main groups did.

Increased diversification rate is in concordance with the evolution of complex morphologies

We inferred significant congruent core shifts in the speciation rate. Overall, 85 congruent core shifts were detected, from which 57 occurred at the MRCA of clades consisting of species with exclusively pileate morphologies. We also performed ancestral character state reconstruction of the main fruiting body types, and we found that the complex morphologies (pileate, gasteroid, coralloid) evolved in the Jurassic and the proportion of lineages with pileate morphology among Agaricomycetes lineages have been increased. We further examined five character states using trait dependent diversification models. We found that the presence

of cap, the enclosed development, the presence of universal and/or partial veil, and the increased hymenophore surface area significantly increased the diversification rate of species.

Developing a low-input tissue-specific RNA-seq workflow

First, we examined the early developmental and tissue formation of *C. cinerea* by inspecting more than 200 cryo-sections. Then, we worked out an RNA sequencing coupled with laser capture microdissection (LCM) workflow by adapting and modifying LCM and RNA-seq methods to *C. cinerea*. We tested the effect of different RNA protecting agents, adjusted cryo-sectioning parameters to reach high quality sections, and tested many cell-disruption methods to increase the yield of RNA extraction. Nine tissue types and seven developmental stages were selected at the early development of *C. cinerea* to be involved in our RNA-seq study and we produced 84 cDNA libraries representing all the tissue types with 2-4 biological replicates.

Potential genes which play role in the evolution of complex multicellularity (transition from vegetative mycelium to hyphal knot stages)

First, we performed differential expression analyses between vegetative mycelium and hyphal knot stages to disentangle the transcriptome changes through the first steps of the mushroom

development. We detected 1,293–2,590 upregulated genes in the tissues of primary and secondary hyphal knots. We found that cell division and cell wall remodeling genes are enriched and could have a pivotal role in the transition from vegetative mycelium into the complex multicellular hyphal knot. Overall, 161 genes were upregulated in the first two developmental stages which could be associated with cell division and hyphal growth pattern including Cyclins, STRIPAK proteins, septins. We found that the number of these genes were increased through the first development steps. We also found 112 upregulated genes related with cell wall biogenesis and remodeling, among them there were 75 genes with putative glycoside hydrolase (GH) activity.

Tissue enhanced genes were detected

We detected genes in a tissue that showed significantly higher expression than the average expression of all tissues of the given stage using deviation contrast in a DE analysis. In this study we focused on tissues related to the morphological traits showed influence on the diversification rate of species. We detected 2,053, 1,053, 1,260 and 941 enhanced genes in the universal veil, lamellae, cap and the partial veil of either stage, respectively. We hypothesized that one of the main functions of the universal and partial veil as well as cap tissues is to give protection for the developing primordium. Based on manually curated functional

annotation lists, we found that defense-related genes represented in the universal veil and cap tissues by a higher portion than in partial veil or lamellae. Many defense-related genes showed a pattern where high expression was exhibited in tissues close to the environment.

Both genetic predisposition and derived genes could drive the evolution of mushroom tissues

We tried to unravel the connection between phenotypic and genetic evolution by examining the level of conservation of tissue enhanced genes using genomic phylostratigraphic analysis. We found that most of the tissue enhanced genes evolved in dikaryotic or ancient fungal and eukaryotic ancestors. This means that tissue enhanced genes predated the evolution of complex structures, and the evolution of tissues could have mainly been driven by exaptation. An exception was an enriched set of genes among universal veil enhanced genes that evolved at the stem node of the Marasmioid clade. Overall, our results suggest that the phenotypic evolution of *C. cinerea* could be driven by mainly ancient and partially young genes as well.

List of publications

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The two publications which constituted of the doctoral degree procedure

Varga T., Krizsán K., Földi C., Dima B., Sánchez-García M., Sánchez-Ramírez S., … Nagy LG. 2019. **Megaphylogeny resolves global patterns of mushroom evolution**. *Nature Ecology & Evolution*, *3*, 668–678. doi: https://doi.org/10.1038/s41559-019-0834-1. IF: 12,541

Nagy LG., Varga T., Csernetics Á., Virágh M. 2020. **Fungi took a unique evolutionary route to multicellularity: Seven key challenges for fungal multicellular life**. *Fungal Biology Reviews*, xxxx. https://doi.org/10.1016/j.fbr.2020.07.002. IF: 4,806

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