Comparative genomics reveals the origin of fungal hyphae and multicellularity

Ph.D. Thesis

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Introduction

Multicellularity evolved independently over 25 times from unicellular ancestors during evolution. The evolution of multicellularity is considered one of the major transitions in the history of life with the first evidences dating back to 3-3.5 billion years. Certain lineages got more and more complex during evolution, and one of the biggest questions of life is why and how this complexity arose and increased in different species. The formation of multicellular body structures can be the result of different kinds of evolutionary innovations. Each lineage where multicellularity has evolved represents a unique solution to the challenges of multicellular organization, as a result of diverse selection and the genomic environment. Eukaryote lineages can acquire multicellularity via either clonal or aggregative routes, which differ in how multicellular precursors emerged by adhesion, cooperation, communication and functional diversification of cells. Besides lineage-specific solutions which resulted in a great morphological diversity, the formation of multicellular structures lie on the same bases in different eukaryote lineages. These solutions are the size advantage, programmed cell division and the differentiation of soma and the early-isolated germ line. These preconditions opened the door for the evolution of adhesion, intercellular communication, cell- and tissue-differentiation and -proliferation, and the division of labor, the five core innovations that facilitated the transition to multicellularity. This multicellular organization enabled organisms to be much more efficient under certain conditions, and thereby created a huge evolutionary advantage for them in different ecological niches.

Researches of the past few years have revealed that the evolution of multicellularity is associated with adhesion-, cell-cell communication- and cell differentiation-related genes and gene families. Also, that the components of this genetic toolkit responsible for multicellular development originated and diversified at different time points during evolution. These investigations could not have happened without the extensive genome sequencing projects and the genomic studies of several non-model organisms. Performing comparative genomic analyses on the newly sequenced genomes of unicellular protists (choanoflagellates, filastereans, ichthyosporeans), which are the closest relatives of metazoans and fungi, was crucial for the investigation of the origin and evolution of multicellularity.

For a long time, it has been assumed that the increase in complexity may have been achieved as a result of a series of major evolutionary transitions, such as the formation of chromosomes, the compartmentalization of eukaryotic cells or the evolution of multicellularity. However, doubts have been raising about this view, and recent studies show that the transition to multicellularity was not as major of a genetic leap as we previously thought. Several evidences support these assumptions. Firstly, the major transitions are considered to be rare evolutionary events. However, multicellularity evolved independently at least 25 times during evolution within different phylogenetic groups. Furthermore, in algae, myxobacteria and the cellular slime mold this transition is relatively easy, and the development of multicellularity is quickly inducible in response to environmental stimuli. The repeated transitions from unicellular to multicellular forms in the fungal kingdom and the fact that complex multicellular fruiting bodies evolved about a dozen times during fungal evolution could also mean, that the hurdles accompanied with this change were not that big as we previously thought.

Recent comparative genomic studies on multicellular species and their unicellular relatives revealed that key multicellularity-related genes – that are previously thought to be exclusive to multicellular organisms – were already present in their unicellular ancestors, and that these conserved multicellularity-related gene families co-opted and evolved for novel functions during the evolution of multicellularity. The discoveries that many requirements for multicellular development likely existed in ancestral unicellular precursors also raise the question, whether this transition can be considered truly as a major evolutionary event. Based on these observations, a hypothesis was formulated by Grosberg and Strathmann, that the transition to multicellularity might not represent a major evolutionary event but a relatively easy minor-major transition.

Among the three big eukaryotic kingdoms, where multicellular forms dominate among extant species, fungi represent a markedly different route to evolve multicellularity. Whilst most multicellular lineages can be recognized as either clonal or aggregative by comparisons to their unicellular relatives, fungal multicellularity might represent a third route to multicellularity by evolving characteristic structures – so called hyphae - of fungal

multicellularity. These thin, tubular structures grow in a polarized fashion and form fractal-like mycelial networks and multicellular hyphal thalli. These mycelial networks have a supporting role in the invasion of substrates for nutrients by saprobes, pathogens and symbionts, and also have key roles in fungal reproduction. Filamentous fungi have an enormous impact not only on terrestrial life and ecosystem functioning, but also on agriculture, biotechnology and human health. The investigation of hyphal growth, and the new discoveries related to this topic are quite important, and play a crucial role in conservation, biotechnology, prevention and treatment of infectious diseases caused by filamentous fungi.

Objectives

In the last decade we have entered the "genomic era". The improvement of genome sequencing technologies enabled us to sequence hundreds of genomes and produce enormous and diverse genomic data. The aim of my work was to answer the arising questions about the evolution of hyphal multicellularity and hyphal morphogenesis using cutting edge bioinformatics approaches, especially comparative genomic methods.

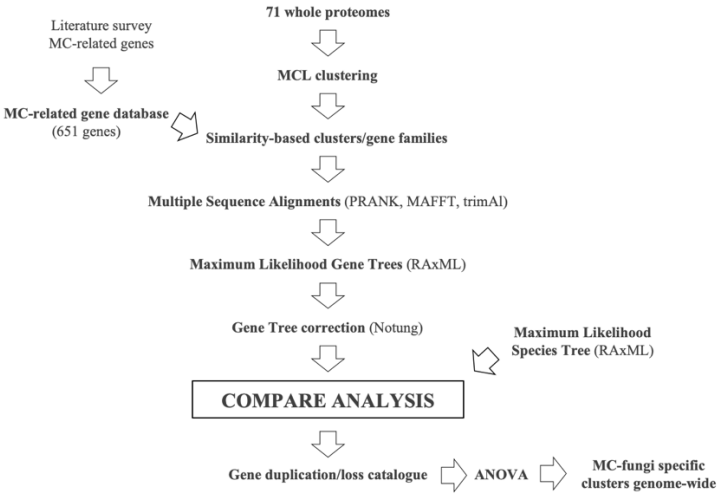
Our knowledge on the evolutionary origin of hyphal multicellularity is far from complete. Nevertheless, the investigation of extant fungal genomes enables us to perform evolutionary genomic analyses and examine the evolution of hyphal multicellularity. With the help of the newest computational techniques, I reconstructed historical patterns of known hypha morphogenesis-related gene families. I also performed systematic investigation of fungal genomes with the aim to seek for gene families whose evolution correlates with the evolution of hyphal multicellularity. Using an extensive taxon sampling I investigated the genomic and developmental commonalities among multicellular lineages and answer how hyphal multicellularity differs from other types of multicellularity.

During my doctoral thesis, my main goal was to address the following questions:

1. What are the evolutionary origins of hyphal multicellularity?
2. Is there any major genetic innovation behind the transition from unicellular to multicellular fungi?
3. Is there any ancient genetic toolkit that plays a role in hyphal multicellularity?
4. What are the functional and molecular predispositions to hyphal multicellularity?
5. How does hyphal multicellularity differ from other types of multicellularity in other lineages?

Methods

I. Taxon sampling
II. Maximum Likelihood species tree estimation
III. Ancestral character state reconstruction
IV. Literature survey for hyphal multicellularity-related genes
V. Analysis of gene family evolution using COMPARE pipeline:



VI. Enrichment analysis of gene duplication events
VII. COMPARE analysis of key metazoan multicellularity-related gene families
VIII. COMPARE analysis of phagocytosis-related gene families
IX. Genome-wide screen for novel hypha morphogenesis-related gene families
X. Analyses of gene losses in yeast-like fungi

Results

Hyphae evolved in early fungal ancestors

To understand the evolutionary origin of multicellular hyphal growth, a species phylogeny was constructed by maximum likelihood analysis, using publicly available genomes. This phylogeny represents 71 species, including 4 unicellular, 39 hyphal, 15 secondarily simplified unicellular (yeast-like) fungi, as well as 13 non-fungal relatives. All of the 71 species in the phylogeny were scored based on their multicellular hypha-forming capabilities. Applying this scoring an ancestral character state reconstruction was performed across the phylogeny to infer the growth mode of fungal ancestors, and the putative origin of hyphal multicellularity and hyphal growth. Results of the reconstruction suggested that hyphal multicellularity evolved from unicellular precursors in some of the earliest fungal ancestors somewhere at the split of Blastocladiomycota, Chytridiomycota and Zoopagomycota lineages.

No expansion of kinase, receptor and adhesive repertoires in fungi

Cell-cell communication and adhesion pathways are key components assumed to be responsible for increased complexity. This concept often correlates with expanded repertoires of genes encoding kinases, receptors and adhesive proteins. In order to investigate whether these kinds of genetic innovations that were found to be key contributors to metazoan multicellularity are also key contributors to hyphal multicellularity, comparative phylogenomic analyses were performed on different signaling system components such as Ser/Thr kinases, hybrid histidine kinases, G protein-coupled receptors and also proteins related to adhesion. The evolutionary analyses of these gene families revealed no significant expansions in filamentous fungi. According to these results, the evolution of Ser/Thr kinase, histidine kinase, GPCR and adhesive protein repertoires highlight important differences between fungal and animal multicellularity and show that the diversification of ‘classic’ metazoan multicellularity-related gene families can’t explain the multicellularity in hypha-forming fungi.

Evolution of hypha morphogenesis-related gene families

To understand the genetic background of the evolution of hyphae, we reconstructed the evolutionary history of 362 known hypha

morphogenesis-related gene families by mapping gene duplications and losses onto our 71-species phylogeny, using the COMPARE pipeline. The investigation of gene family evolution mainly focused on gene duplication events at the origin of hyphal growth.

The evolutionary history of hyphal morphogenesis-related gene families showed a mixed picture. 50% (181) of the gene families were older than hypha morphogenesis itself. Significant proportion (71%, 128) of these gene families was ancient and conserved across all eukaryotes with very few or no duplication events at the origin of hyphal multicellularity. Another subset of these "old" gene families comprises families that have deep eukaryotic origins, but also show elevated duplication numbers coincident with the evolution of hyphae. In the second category, there are gene families whose origin mapped to the origin of hyphal growth. Total 4.7 % (17) of gene families coincide with the evolution of hypha morphogenesis. These gene families could have evolved *de novo* at the origin of hyphal growth representing evolutionary innovations related to the transition to hyphal multicellularity, or diverged so much that sequence homology is not detectable any more across species. The third category represents newly evolved "young" gene families. These families originate after the emergence of hyphal growth and cover almost the other half (45.3% (164)) of the investigated gene families, and they are regarded as lineage and species-specific genetic innovations related to the evolution of hyphal growth. Overall 25.7% (93) of the investigated gene families showed duplications at the origin of hyphae, and these gene duplication events were characteristic mainly in "cell wall biogenesis and remodeling" and "transcriptional regulation" categories. However, separate statistical analyses on morphogenesis-related gene families revealed no individual gene families having significantly elevated duplication rate at the origin of hyphal multicellularity. These observations indicate a lack of a major burst of gene family origin coincident with the evolution of hyphae, and also limited novelty by gene duplication events in regard to hyphal multicellularity.

Structural properties of hypha morphogenesis-related genes

To investigate whether changes in structural properties of hypha morphogenesis-related genes show a correlation with the evolution of hyphal multicellularity, we investigated changes in gene architecture

(e.g. intron and coding sequence length), using two-tailed Welch's t-test. Our comparative analyses on gene structures revealed significant differences between hypha morphogenesis-related genes of unicellular and multicellular fungi, and showed that several modifications of CDS and intron length in multicellular fungi, although individually are small changes, but they could have contributed to the evolution of hyphal multicellularity. Significant differences ($p < 0.05$) were observed in gene, coding sequence (CDS) or intron lengths between unicellular and multicellular fungi in 7 out of 10 functional groups (exceptions are "cell cycle", "signaling" and "transcription" categories). Coding sequences of "septation" and "polarity" genes were significantly longer in multicellular than in unicellular fungi ($P = 0.0017$; $P = 0.00012$). An opposite pattern was observed in introns, which were on average longer in unicellular fungi in actin cytoskeleton-, polarity-, septation-, and vesicle transport-related genes.

Phagocytotic genes were exapted for hypha morphogenesis

Our set of hypha morphogenesis genes included several entries associated with the process of phagocytosis in non-fungal eukaryotes. This is surprising given that phagocytosis is not known in fungal species, as their rigid cell wall forms a physical barrier to it. We therefore examined the evolutionary history and fate of 414 phagocytosis-related gene family in multicellular fungi based on the phagocytotic machinery of *Dictyostelium discoideum* and other eukaryotes. COMPARE analysis of these gene families revealed, that several phagocytotic genes are conserved across fungi, despite the loss of phagocytosis itself. These results highlight exaptation as another mechanism for the recruitment of genes for hyphal growth in multicellular fungi. Also, these findings suggest, that the ancient genetic toolkit responsible for phagocytosis in *Dictyostelium* and other eukaryotes possibly adapted during fungal evolution for serving other purposes in hyphal growth and multicellularity.

Genome-wide screen finds novel gene families linked to hypha morphogenesis and hyphal multicellularity

To answer, whether there are further gene families with potential connection to hyphal multicellularity, a systematic genome-wide search was performed for other hyphal multicellularity-related gene

families using ANOVA ($p < 0.05$). It was reasoned that gene families underlying hyphal multicellularity should originate or diversify at the origin of hyphal growth and be conserved in descendent filamentous fungi. The analysis predicted a link to hyphal growth for another 414 families. Out of the 414 families, 114 originated at the emergence of hyphal multicellularity, while the others showed increased duplication rates that exceeded the expectation derived from genome-wide figures of gene duplication. This search resulted in already known morphogenetic families but also uncharacterized ones with no information about their functions and roles in hyphal growth. These unknown gene families can serve as potential candidates for further functional studies and characterization, revealing additional details about the evolution of hyphal growth.

Yeasts retain genes required for hypha morphogenesis

Yeast-like fungi are secondarily simplified organisms with reduced ability to form hyphae. The ancestral character state reconstruction performed on the 71 species implies that yeasts derived from filamentous ancestors, and thus they represent a classic example of the reduction in complexity. They were hypothesized to have lost the capability to form multicellular filaments, even though rudimentary forms of hyphal growth (termed "pseudohyphae") exist in most yeast species. To investigate whether this morphological reduction was associated with genomic changes, we analyzed the fate of hypha morphogenesis-related genes in five yeast-like lineages.

The analysis of gene losses in five yeast-like lineages revealed that 54-65% of hypha morphogenesis-related genes were retained in yeasts species, suggesting that the hyphal morphogenesis-related genes are in general less dispensable for yeasts than are genes with other functions. The finding that yeast species possess a relatively big proportion of the genetic toolkit responsible for hyphal growth, can be a possible explanation for why yeast-like species are able to produce elongated filaments, pseudohyphae or sometimes true hyphae in response to different kinds of external stimuli (e.g. presence of serum, deprivation of nutrients etc.).

Discussion

In summary, the extensive co-option and exaptation of ancient eukaryotic genetic toolkits may have been the most important driving

forces of the evolution of hyphal multicellularity, followed by gene duplication events at the origin of hyphal growth, and changes in gene structures, accompanied by only limited *de novo* gene family birth. These novel findings related to hyphal multicellularity in fungi mirror patterns of the evolution of other multicellular organisms, such as animals and plants, which gave rise to the hypothesis that in terms of genetic novelty, transitions to multicellular life represent a minor rather a major evolutionary step. Also, our observations suggest that the prerequisites for the development of hyphal multicellularity differs considerably from that of other lineages. These differences raise the possibility that in addition to the aggregative and clonal modes of evolving multicellularity, hyphal multicellularity should be considered a third, qualitatively different route to multicellular development.

Publications

Mandatory peer-reviewed international publication on which this thesis is based:

Kiss, E., Hegedüs, B., Virágh, M., Varga, T., Merényi, Z., Kószó, T., Bálint, B., Prasanna, AN., Krizsán, K., Kocsubé, S., Riquelme, M., Takeshita, N., Nagy, GL, "Comparative genomics reveals the origin of fungal hyphae and multicellularity", *Nature Communications 2019 Sep 9;10(1):4080*

Mandatory peer-reviewed international publication for the fulfillment of doctoral process:

Miyauchi, S., **Kiss, E**., Kuo, A., Drula, E., Kohler, A., Sánchez-García, M., Andreopoulos, B., Barry, K.W., Bonito, G., Buée, M., Carver, A., Chen, C., Cichocki, N., Clum, A., Culley, D., Crous, P.W., Fauchery, L., Girlanda, M., Hayes, R.D., Kéri, Z., LaButti, K., Lipzen, A., Lombard, V., Magnuson, J., Maillard, F., Morin, E., Murat, C., Nolan, M., Ohm, R. A., Pangilinan, J., de Freitas Pereira, M., Perotto, S., Peter, M., Pfister, S., Riley, R., Sitrit, Y., Stielow, B.J., Szöllősi, G., Žifčáková, L., Štursová, M., Spatafora, J.W., Tedersoo, L., Vaario, L.M., Yamada, A., Yan, M., Wang, P., Xu, J., Bruns, T., Baldrian, P., Vilgalys, R., Dunand, C., Henrissat, B., Grigoriev, I.V., Hibbett, D., Nagy, GL., Martin FM, "Large scale genome sequencing of mycorrhizal fungi provides insights into the early evolution of symbiotic traits", *Nature Communications 2020*

Other peer-reviewed international publications:

Nagy, GL., Tóth, R., **Kiss, E.**, Slot, J., Gácser, A., Kovács, GM, "Six Key Traits of Fungi: Their Evolutionary Origins and Genetic Bases", *Microbiol Spectrum 5(4):FUNK-0036-2016.*

Book Chapter:

Nagy, GL., Tóth, R., **Kiss, E.**, Slot, J., Gácser, A., Kovács, GM, "Six Key Traits of Fungi: Their Evolutionary Origins and Genetic Bases" In: Heitman J, Howlett BJ, Crous PW, Stukebrock EH, James TY, Gow NAR (szerk.) The Fungal Kingdom. New York: American Society for Microbiology Press (ASM), 2017. pp. 35-56. (ISBN: 9781555819576)

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