

Using evolutionary genomics, transcriptomics, and systems biology to reveal gene networks underlying fungal development

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Highlights

- Diverse model species contribute to research on the evolution of fungal development.
- Transcriptomics facilitates study of developmental evolution from non-model fungi.
- Evolution of expression underlying evolved phenotypes can guide functional analysis.
- Bayesian networks predict promising experiments using integrative systems biology.
- The future of fungal evo-devo lies with systems biology of developmental traits.

Abstract: Fungal model species have contributed to many aspects of modern biology, from biochemistry and cell biology to molecular genetics. Nevertheless, only a few genes associated with morphological development in fungi have been functionally characterized in terms of their genetic or molecular interactions. Evolutionary developmental biology in fungi faces challenges from a lack of fossil records and unresolved species phylogeny, to homoplasy associated with simple morphology. Traditionally, reductive approaches use genetic screens to reveal phenotypes from a large number of mutants; the efficiency of these approaches relies on profound prior knowledge of the genetics and biology of the designated development trait—knowledge which is often not available for even well-studied fungal model species. Reductive approaches become less efficient for the study of developmental traits that are regulated quantitatively by more than one gene via networks. Recent advances in genome-wide analysis performed in representative multicellular fungal models and non-models have greatly improved upon the traditional reductive approaches in fungal evo-devo research by providing clues for focused knockout strategies. In particular, genome-wide gene expression data across developmental processes of interest in multiple species can expedite the advancement of integrative synthetic and systems biology strategies to reveal regulatory networks underlying fungal development.

Keywords: fungi; development; evolution; systems biology; transcriptomics; genomics

1. Introduction

“Morphology was studied because it was the material believed to be the most favorable for the elucidation of the problems of evolution...” (Bateson, 1922)

Developmental biology, one of the most longstanding and deeply examined subdisciplines in biology, reveals how complex life forms are constructed by the growth, repositioning, structure, and identity of cells and tissues. Examination of morphological development has been vigorous in almost every major organismal group, and knowledge of developmental biology has accumulated along with advances in cell biology, developmental genetics, and the systems biology. Nevertheless, we remain ignorant of some of the basic elements of how a body shape is planned and formed in response to genetic and environmental signals—even in well studied models.

Evolutionary biology and developmental biology have long been considered complementary disciplines, and their synthesis has recently been boosted by their merger into the exciting field of evolutionary developmental biology (a.k.a. Evo-Devo)—research that investigates the developmental processes and the evolution of those processes among different organisms (Hall, 2012). Among the oldest models in cell biology, fungi have contributed significantly to evolutionary developmental biology and in recent times continue to serve as highly tractable workhorses for genetics and genomics, and now systems biology revolution (Bennett and Arnold, 2001).

1.1 Fungal diversity and fungal development

Fungi represent one of the most diverse organismal groups on earth in terms of their ecology, their ubiquity, and their manifold morphological forms. Among eukaryotes, fungi are relatively simple in terms of their cell and tissue types, along with high genome diversity accompanying their wide ecological distribution (Alexopoulos, 2007; Blackwell, 2011; Mohanta and Bae, 2015; Stajich et al., 2009; Taylor et al., 2017; Zeng et al., 2017). Fungal development encompasses a very wide range of complexity, ranging from unicellular yeasts that can be found in flowers and guts of beetles and wasps (Lachance et al., 2013; Stefanini et al., 2012), to the largest known fruiting body of a wood decaying fungus—weighing nearly 500 kg (Dai and Cui, 2011), to postfire fungi that blossom quickly and transiently after forest fires (Glassman et al., 2016), to the oldest known mycelium—over 1500 years in age and 15 ha in occupied area (Smith et al., 1992). In addition, morphological divergence, which in fungi commonly includes the evolution of morphologies of highly reduced complexity, can make it challenging to identify orthologous structures among even closely related lineages (Wang et al., 2016). Many higher fungi produce elaborate sexual reproductive structures predominantly for survival during their sexual life cycles, but during their asexual cycle these same fungi produce simple but vigorous structures that generate myriad, short lived asexual spores for rapid dispersal. Convergent and

divergent evolution of phenotypes are not rare in fungi (Alexopoulos, 2007; Nagy et al., 2014; Shang et al., 2016; Torruella et al., 2015), often resulting from diverse ecological pressures on a limited set of available simple reduced morphologies. For example, many leaf endophytic fungi produce dark-colored, covered, small fruiting bodies, while their saprotrophic relatives produce bright-colored, exposed, large fruiting bodies (Ruibal et al., 2008; Wang et al., 2009). Like plants and animals, some fungi—despite their more limited palette of cell and tissue types—have evolved structures for specific functions that are not common to other fungi. Remarkable examples of these innovative morphologies include the specialized and diverse penetration structures (appressoria) present in many plant pathogenic fungi (Ames, 2017; Geoghegan et al., 2017; Mendgen et al., 1996), the mycorrhizal nutrient bridges that develop between mushroom-forming fungi and their associated plants (Bravo et al., 2017; Iwaniuk and Błaszczowski, 2014; Jiang et al., 2017; Marks, 2012), differentiation of wood decay models among higher fungi (Floudas et al., 2012; Hibbett and Donoghue, 2001; Nagy et al., 2016), and the trapping rings, nets and adhesive hyphae unique to the nematode-trapping fungi in the Orbiliomycetes (Hyde et al., 2014).

1.2 Fungal models for developmental biology

Fungal species have supplied model systems that have advanced multiple disciplines. Many of the fungi selected and developed as model systems share similar features: fast growth, short life cycle, manipulability in a laboratory setting, and distinctive morphology facilitating identification. Because these traits are not unusual in fungal species, the model species are often also of direct economic importance. The best-known and most well-studied experimental fungal model species are the yeasts *Saccharomyces cerevisiae* (bread and wine yeast) and *Schizosaccharomyces pombe* (Egel, 2013; Rose, 1981), which are important tools in molecular genetics, as well as human associated *Candida* species (Ene et al., 2016; Prasad, 2017). As single-celled eukaryotes, these yeasts serve as powerful model systems for the investigation of numerous fundamental principles and mechanisms, including but not limited to cell-cycle control, mitosis and meiosis tool kits, genome organization, epigenetics and epigenomics, DNA recombination and repair, signal transduction, population genetics, and the aging process (Boynton et al., 2017; Caudy et al., 2017; Egel, 2013; Fuchs and Quasem, 2014; Salehzadeh-Yazdi et al., 2014; Tsubouchi, 2006). A global genetic interaction network based on *S. cerevisiae* provides a wiring diagram of cellular function (Costanzo et al., 2016). Yeasts also are models for developmental biology, especially for cell-to-cell communication, cell polarity, and budding and mating processes, although the scope of their contributions has been restricted to cellular development (Drubin, 1991; Liti, 2014; Tomićić and Raspor, 2017; Winters and Chiang, 2016). Representatives of long-time multicellular fungal models for developmental biology include the ascomycetous species *Aspergillus nidulans* (Braus et al., 2002; Croft, 1966; Seo, 2005; Timberlake, 1993) and *Neurospora crassa* (Aramayo and Selker, 2013; Davis, 2000; Davis and Perkins, 2002; Mitchell, 1955; Selker, 2017) and their closely related species, and basidiomycetous species *Schizophellum commune* (Casselton and Kües, 2007; Essig, 1922;

Wessels, 1989), *Coprinopsis cinerea* (Plaza et al., 2014), and *Coprinus comatus* (Casselton and Kües, 2007; Junjie-Yuan et al., 2010; Winterboer and Eicker, 1983). Among these multicellular models, *N. crassa* was the first one whose genome was sequenced, a tribute to its longstanding position as a model for genetics, beginning with its role in the formulation of the “one gene--one enzyme” hypothesis proposed by Edward Tatum and George Wells Beadle (1941), who—for this insight—won the Nobel Prize in Physiology or Medicine in 1958 (Davis and Perkins, 2002). Unlike yeasts and *Aspergillus* species, *N. crassa* has little importance in medical, agriculture and food industries—although it is consumed as ontjom (Beuchat, 1976), and spurred by its model status, some have investigated its potential in biofuel development and biotechnology (Benz et al., 2014; Roche et al., 2014; Seibert et al., 2016; Tian et al., 2009). Studies of the developmental biology of *N. crassa* have been mainly focused on asexual development, although over many years, numerous sexual development- and meiosis-related genes have been characterized (Borkovich et al., 2004; Iyer et al., 2009; Krystofova and Borkovich, 2006; Lehr et al., 2014; Springer, 1993; Springer and Yanofsky, 1992; Stajich, 2014; Vigfusson and Weijer, 1972; Wang et al., 2014, 2012).

1.3. Knowledge of fungal development contributes to basic and applied sciences

Compared with most plant or animal models, the genomes of fungal model species are comparatively small and simple. Nevertheless, they retain genes for most of the core metabolism, conserved pathways, and cellular developmental regulatory mechanisms in eukaryotes (Brown, 2006). For research at the cellular development level, yeast definitely exemplifies “a model for all eukaryotic biology derives from the facility with which the relation between gene structure and protein function can be established” (Botstein and Fink, 1988, 2011). Early genetics in yeast models reaped benefits from approaches pioneered to generate biochemical mutants in *Neurospora*, which had been a model to study nitrogen, sulfur, and phosphate metabolism for eukaryotes seven decades ago (Beadle and Tatum, 1941), and more recently a model for understanding recombination, differentiation (Borkovich et al., 2004), silencing and DNA methylation (Honda et al., 2016), morphogenesis and cell biology (Kronholm et al., 2016), and circadian rhythms (Borkovich et al., 2004; Dunlap, 2008). Circadian rhythm studies in *N. crassa* resulted in the cloning of the first clock genes in the early 1980s (Dunlap et al., 2007). Hall and Rosbash later identified the *period* gene from fruit flies, work that led to a recent Nobel prize shared by Hall, Rosbach, and Young. Discovery of the fungal circadian system occurred more than a decade prior to the cloning of the first human clock genes (Antoch et al., 1997). With the traditional gene by gene approach, a community of hard-working *Neurospora* geneticists contributed knowledge of 1,100 genes with phenotypes over 60 years, before the recent high-throughput approach that produced 11,000 knockout strains providing a means to knowledge of function in over 9600 genes (Collopy et al., 2010). Recent studies on *Neurospora* and *Aspergillus* shed light on how internal clock-related oscillations and asexual development are regulated in response to environmental signals in fungi (Dasgupta et al., 2015; Hong et al., 2014; Montenegro-Montero et al., 2015; Rokas, 2013). Fungal models have also been used to

understand how sex may have evolved in eukaryotes (Heitman, 2015). Many of the core methods of genomics and systems biology applied to eukaryotic systems were first developed from fungal genetics, including physical mapping, pulsed field gel electrophoresis, knockout collections, signature tagged mutagenesis, genome assembly methods, transcriptomic profiling, proteomic profiling, and genome-scale identification of protein-DNA interactions (Kück, 2013).

Other fungal models have also been examined to illuminate fungal multicellular developmental biology. For example, whether an “hourglass” model of the conservation of expression of developmentally-related genes appears in fungi like it appears in other eukaryotes (Drost et al., 2015; Kalinka et al., 2010; Ninova et al., 2014; Quint et al., 2012) was investigated using the model mushroom-forming fungus *Coprinopsis cinerea* (Cheng et al., 2015).

Aspergillus species have been intensively studied for their asexual growth—mainly because secondary metabolic products, such as mycotoxins, antibiotics and citric acids, are enriched during late asexual development (Adams and Yu, 1998; Bayram et al., 2016; Garzia et al., 2013; Keller, 2006; Yin et al., 2013). Some fungi do not form fruiting bodies in laboratory conditions, implying that unknown environmental factors are involved in the growth and development in these fungi. For instance, metagenomic evidence suggests that the composition of bacterial communities is associated with the differentiation and development of sexual structures in black truffle fungus (Antony-Babu et al., 2014). Once the genome sequencing of *Neurospora crassa* was complete (Galagan et al., 2003), genome sequences of *Aspergillus* species (Galagan et al., 2005; Machida et al., 2005; Nierman et al., 2005; Ronning et al., 2005; Yu et al., 2005), *Magnaporthe oryzae* (Dean et al., 2005), and *Fusarium graminearum* (Cuomo et al., 2007) quickly followed. These core filamentous ascomycete species established a jumping board to study previously intractable species and elucidate host-pathogen interactions in both human and plants, development of infection-associated structures, and hyphal development and sporulation, both as conidia and sexual fruiting bodies. These allowed us to move to study of development and sporulation in the obligately biotrophic pathogens such as rusts (Cuomo et al., 2007; Yin et al., 2015) and powdery mildews, and to determine the adaptive genome evolution of *Malessizia*, the human skin pathogen in the Ustilaginomycotina, a subphylum of predominantly plant smut fungi (Wu et al., 2015). Study of fungal development has led to the realization that regulation of secondary metabolite production and development are correlated (Keller et al., 2005), and the global regulator complex, VelB/VeA/LaeA, that coordinates light signaling with development also regulates secondary metabolism through chromatin remodeling mediated by LaeA (Bayram et al., 2016). Very recently, regulators of the stages of asexual sporulation have been shown to also regulate the genes for secondary metabolites that accumulate during those stages of development (Lind et al., 2017).

2. Study of fungal development and its evolution.

Development of fungi has received considerable attention in the form of high-level analyses of the systematics and classification of fungi, that map evolutionary developmental characteristics to taxonomy. Indeed, from early 17th century, most studies on fungal development and its

evolution had to depend solely on morphology and anatomical evidence (Bary et al., 1887; Drews, 2001), eventually including ultrastructural characters. Because of this confounding dependence, relationships between morphological development and evolution were long considered as a black box that could not be directly investigated. However, longstanding knowledge from morphological studies continues to supply ideas, hypotheses, and research questions about fungal development that now can be investigated using molecular probes. Consequent advances in fungal phylogeny and phylogenomics have dramatically improved our knowledge about relationships among various fungal lineages and within taxa. These solved phylogenies make it possible to precisely reconcile the evolution of morphological diversity and genetic diversity in fungi (Dornburg et al., 2017; McCarthy and Fitzpatrick, 2017; Nagy and Szöllősi, 2017; Zhang et al., 2017) .

2.1 Traditional gene by gene approaches to the evolution of development

Significant effort and resources have been spent performing genetic perturbations to reveal developmental phenotypes in various fungal models, and important breakthroughs have been extensively reviewed (Chiu and Moore, 1996; Esser and Lemke, 1994; Kües and Fischer, 2006; Lehr et al., 2014; Meinhardt and Wessels, 2013; Nowrousian, 2014; Osiewacz, 2002; Sikhakolli et al., 2012; Trail, 2013; Wendland, 2016). Sequenced genomes of fungal models have recently accelerated the molecular identification of genetic markers phenotypically identified via traditional mutant screens (Riquelme and Martínez-Núñez, 2016). In one example, McCluskey et al. (2011) examined 18 classical mutant strains in *Neurospora crassa*, readily identifying all of them to their causative DNA mutation using whole genome sequencing. Genome sequencing and annotation of increasing numbers of non-model species has also enabled technologies that facilitate the investigation of molecular developmental biology in non-model organisms. Nevertheless, reductive gene by gene approaches to assess genetic basis of development processes have been limited by several issues: genes may have multiple phenotypes, including fatal ones; several genes may have redundant functions for certain developmental phenotypes or in certain environments; discovery of phenotypes for individual genes may require specific genetic and environmental settings that are not easily known; and lastly, the development of complexity and its regulation is widely believed to involve interactions of many genes through gene-networks that are sparsely revealed by associations detected between single genes and single developmental phenotypes (Cheng et al., 2013; Le Nagard et al., 2011; Li and Johnson, 2010; Macía et al., 2011; Payne and Wagner, 2014). In addition, one of the biggest hurdles to finding genes involved in a particular process is the selection of the genes that are to be functionally assayed: candidate gene lists are often short to nonexistent, as well as challenging to prioritize. Prediction is critical, because molecular genetic assays entail extensive effort, in filamentous fungi requiring recovering a single genetically pure cell for each putative manipulation. Depending on the gene expression effects that one is trying to investigate, one must also choose among diverse potential experiments, such as directed mutation to abrogate function, knockout, knock-down, and knock-up experiments. To phenotype putative mutants for

complex traits such as fruiting body development, each putative transformant must be grown under conditions that induce fruiting bodies. Then the fruiting bodies must be examined (by sectioning and microscopy) to determine whether development has been affected (Collopy et al., 2010; Colot et al., 2006; Fu et al., 2011; Lord and Read, 2011; McCluskey and Plamann, 2008; Teichert et al., 2012; Trail, 2013).

2.2 Evolutionary genomics and transcriptomics of fungal development

Genome-wide approaches have recently exhibited promise in resolving some puzzles in fungal development and its evolution, especially developmental traits regulated by genes conserved in ontology (Maheshwari, 2016). Filamentous fungi typically feature about 15,000 genes; knocking them all out and characterizing them is a monumental task. High throughput systems still require the conscientious examination of individual knockouts throughout the life cycle, and without guidance as to which stages are the relevant ones to look at, highly relevant phenotypes are easily missed. A common strategy for the study of the evolution of development in model organisms begins with identification of possible genes and gene networks that respond to a developmental process (genotyping to phenotyping) in one or several species (models), and then proceeds to identification of conserved elements along the evolutionary history of the developmental traits (Bolker, 2014). In fact, assessment of the components of a gene network underlying a developmental process using genome-wide approaches requires extensive foreknowledge of the genetic basis of the investigated process, usually derived from previous gene-by-gene studies. Detailed genetic knowledge obtained regarding the cell walls of yeast model systems has empowered comparative genomic analyses that contribute fundamental understanding of their evolution in fungi (Ruiz-Herrera and Ortiz-Castellanos, 2010; Xie and Lipke, 2010). A pioneering study combined a time course of genome-wide gene expression on a single species and gene knockout to assess gene function in model *S. cerevisiae* (DeRisi et al., 1997; Díaz et al., 2009; Lashkari et al., 1997). Knockout phenotyping has been used to understanding gene functions in filamentous fungi, especially for many mutants created by target nonspecific approaches, and many of these studies focused on genes known to exhibit similar function or known to be involved in related pathways or networks (Chai et al., 2016; Collopy et al., 2010; Engh et al., 2010; Fu et al., 2014, 2011; Kopke et al., 2010; Lehr et al., 2014; Nowrousian and Cebula, 2005; Schindler and Nowrousian, 2014; Teichert et al., 2016; Watters et al., 2011). Recently, transcriptomic data across sexual development helped to infer a network of meiotic silencing genes, which were individually characterized for function in perithecial development based on their knockout phenotypes in *Neurospora crassa* (Wang et al., 2014). Interestingly, peak expression during wild type perithecial development was perfectly reversed from their genetically determined order of operation in meiotic silencing, indicating a discrepancy between their functional order in wild type development and in the event of meiotic silencing. That match between expression regulation and expected knockout phenotype epitomizes how good practice of a focused knockout strategy can be especially informative. Regulatory networks and their expression are likely conserved for orthologous developmental

traits—especially key developmental characters among species sharing the development processes. Comparing fungal species for their genomics and transcriptomics consensus during the development of the same traits has been used to infer the genetic basis of fungal biology (Chibucos et al., 2016; Gan et al., 2012; Ge et al., 2016), a genomic approach that provides an alternative for identifying genes behind common development traits. *Arboretum*, a program that integrates evolutionary genomics and transcriptomics to assess shared developmental co-expression traits among species, has been applied to yeast groups (Knaack et al., 2016; Roy et al., 2013).

However, fungi exhibit a huge diversity in morphology, and little of this phenotypic diversity can be examined by starting with model species and the integrative approaches mentioned above. In theory, differences in developmental traits among diverse species can be explained by differences in gene regulation and expression that evolved along the taxon phylogeny (Demidenko and Penin, 2012; Kalinka et al., 2010; Shestakova, 2015; West-Eberhard, 2003). Therefore in principle, the candidate gene pool for phenotyping morphological differences on traits can be narrowed down—and prioritized—using comparative genomics and transcriptomics. Differences in expression of orthologous genes along their evolutionary history are likely to contribute to developmental differences among homologous morphological traits. Under this view, reconstruction of ancestral gene expression phenotypes would facilitate identification of genes with the largest evolved increases in gene expression across development, which in turn would likely be involved in relevant phenotypic novelties. Guidance provided by this logic could lead to discovery of relevant genes more efficiently than other gene deletion studies targeting whole genomes or gene families.

Such a strategy for pinpointing genes responsible for the evolution of certain biological processes has recently been developed using the fungi *Neurospora crassa* and *Fusarium graminearum*, models for perithecium development, as well as species closely related to these fungi (Trail et al., 2017). Ancestral gene expression states, including ancestral changes across sexual development, were inferred for five related species during comparable stages of sexual development. An association between genes whose transcription has been substantially and significantly altered during the evolutionary process and phenotypes that change along the same phylogenetic branch was validated by performing functional studies: gene disruption that frequently resulted in substantial changes in fruiting body development in the disruptants at morphological stages when the wild type gene's expression shift was divergent between species (Trail et al., 2017). Many genes that relate to novel phenotypes in the development of fungal fruiting bodies can be predicted and prioritized for focused knockout phenotyping by estimating which genes have increased their expression recently in evolutionary time. To do so, genome-wide gene expression should be measured for distantly related fungal species during the same developmental process, where a highly similar sexual morphology evolved with distinct species-specific characters among these species. Ancestral expression can then be calculated by diverse means (Cooper et al., 2015; Joy et al., 2016; Rohlf et al., 2013), including using a Continuous Ancestral Character Estimation (CACE) that assumes that traits evolve according to a Brownian

motion process (Schluter et al., 1997), a calculation for which there is an available R package, Analyses of Phylogenetics and Evolution (APE; (Paradis, 2011; Paradis et al., 2004). Such a guided knockout approach demonstrated a significantly higher efficiency than traditional systematic knockout approach in identifying genes that may be responsible for observed developmental differences as applied in *Neurospora* and *Fusarium* (Trail et al., 2017). The study demonstrates a precision shortcut for researchers: if they first identify identical genes present across different species that have recently evolved and increased in gene expression, they can knock out or otherwise perturb this smaller targeted set to reveal genes that underlie a phenotype of interest in a specific species (Fig. 1). Predictions provided by the evolutionary analysis of gene expression enabled identification of genes that were likely to exhibit a knockout phenotype involved in any process, even if complex and understudied.

The power of this approach for understudied species lies in the pairing of well-studied organisms with phylogenetically close organisms that are not so well studied. The requirements for inclusion of species are that they must have a genome sequence, and they must be able to be cultured in a common environment, yielding extractable mRNA from homologous stages of development. Although an averaging of mRNAs in organisms that develop asynchronously makes the technique remain applicable to populations that exhibit developmental stochasticity, the results are more powerful for organisms that can be manipulated to develop synchronously (Trail et al., 2017). The process has been demonstrated to be highly effective for gene discovery of conidial germination and plant penetration and can be effective even when conidia are collected while germinating on a diversity of host plants (Trail et al., 2017). Thus, even though inference of the evolution of gene expression is formally compromised by growth on different substrates, it appears that the use of an evolutionary approach on data gathered from heterogeneous but functionally analogous environments can still empower the discovery of genes of functional importance during the developmental process.

This transcriptomic evolution of development approach worked just as well for unannotated genes: among the candidate genes identified for further phenotyping investigation are many whose functional identification has been lacking. In fact, whole genome screening for sexual development mutants had already been performed for *Neurospora*; nevertheless, most the phenotype assignments to *N. crassa* genes reported in Trail et al (2017) are new. While the whole-genome screen can be productive, focused knockout can be a powerful and complementary form of investigation. Moreover, to date only one filamentous fungus had been subject to a whole genome knockout project—such undirected studies are challenging to fund. Trail et al (2017) provide a strategy that can identify targets for knockout and phenotyping in multiple species at once, reducing the effort required to identify genes playing a role in a particular process, and addressing relevant hypotheses regarding the evolution of morphologies of interest. Gathering expression and morphological data confers independent benefits for transcriptomic and evolutionary studies, and provides synergies with knockout phenotype data. It is likely (if not inevitable) that there will be increasing numbers of comparative studies, empowered by genome sequencing and modern knockout technologies, examining the function

of gene knockouts across suites of related species featuring divergent morphologies. Performing these knockouts in a principled and efficient way should be attractive to everyone, but especially to anyone with interest in the evolution of development. This approach is poised to take on a larger and larger role within that field.

3. Conceptual and practical strategies to systems biology of fungal development

While identification of genes to examine can be aided by consideration of evolutionary dynamics of gene expression across phylogenies, refinement of models of those gene interactions within individual species requires a more focused approach. Traditional reductionist approaches of gene-by-gene methodologies are laborious and tend to lead to overly linear description, and genomic inference from comparative genomics and transcriptomic data alone have little power to reveal the details of the complex processes of macroscopic development. Systems biology, an emerging holistic interdisciplinary approach, is a biology-based field of study that focuses on complex interactions within biological systems (Albrecht and Guthke, 2009; Kaneko, 2006; Marcum, 2009). Systems developmental biology has attracted considerable attention and inspired funding opportunities (Perrimon and Barkai, 2011). Systems biology is the integration of many observations to understand the emergent functions of complex adaptive modules. To reveal the complex, dynamic and nonlinear systemic architecture of development, a systems biology approach can be used to incorporate mathematical interval analysis and model discrimination analysis (Benfey, 2011; Boogerd et al., 2007; Ideker et al., 2001). This approach consists of 1) identifying dynamically relevant parts of the system, 2) creation of one or more model hypotheses of the interaction among these parts, 3) application of these models on high-throughput data to initiate a working hypothesis, 4) identification of the most effective perturbation steps to verify and to improve the initial hypothesis, and 5) evaluation and revision of the model hypothesis to refine the set of putative models to be verified with phenotypic data and gene manipulation.

*3.1 Develop criteria to guide systems biology for developmental questions using *N. crassa**

N. crassa has been a productive genetic model organism, with continued promise for revealing the molecular basis of the complex metabolic and morphological changes associated with sexual development of fungi (Bistis et al., 2003; Borkovich et al., 2004; Lord and Read, 2011; Selker, 2011). The *Neurospora* research community also has constructed and made available over 11000 knockout strains for more than 9600 genes (Jay C. Dunlap et al., 2007). Previous research on *N. crassa* has yielded knowledge, data and tools that facilitate the study of the genetic basis of asexual growth and development (Iyer et al., 2009; Krystofova and Borkovich, 2006; Pöggeler, 2011; Rodriguez-Romero et al., 2010; Springer, 1993; Springer and Yanofsky, 1992; Vigfusson and Weijer, 1972; Wang et al., 2014, 2012; Whittle and Johannesson, 2011).

Analyses of gene expression and genetic regulatory networks in diverse model organisms have demonstrated that genes work together in response to regulatory factors to shape metabolic

processes and morphological development as well as aging (Freeman et al., 2012; Guo et al., 2011; Huang et al., 2012; Judge et al., 2017; Tong, 2004). For example, a study just published shows that evolution of the fungal polarization network is highly dynamic but functionally conserved through structural conservation in polarization protein networks (Diepeveen et al., 2017). However, further investigation of any putative network requires hypothetical prediction on the basis of heterogeneous data arising from diverse experimental designs, missing information, and limited prior knowledge (Emmert-Streib and Glazko, 2011; Zarringhalam et al., 2013). One classification of gene networks that can be developed for genome-wide time-series data (Barman and Kwon, 2017; Kim et al., 2004; Park et al., 2017; Zamanighomi et al., 2014) divides them into three categories: gene co-expression networks (GCNs) using continuous data (López-Kleine et al., 2013), gene regulatory networks (GRNs) using discrete data (Hecker et al., 2009), and Bayesian networks (BNs), which allow both data types, thus making it particularly feasible to integrate genetic and environmental factors into predictions (Ziebarth et al., 2013; Ziebarth and Cui, 2016). Regardless of how a gene network model is inferred, prior knowledge of gene associations to be investigated should be gathered from literature and data mining, and some level of experimental validation is always needed (Hecker et al., 2009). Yeast model species are among the best-studied fungal models, and most recent knowledge about fungal metabolism pathways and cellular development networks has been revealed using yeast models (Mustacchi et al., 2006). The complex interactions between fungi and their hosts have also been the subject of extensive experimental investigation. An early genome-wide GRN network model revealed interactions between host and fungal pathogen *Candida albicans* (Altwasser et al., 2012) and *Aspergillus fumigatus* (Guthke et al., 2016). Unlike GCNs and GRNs that use comparatively simple strategies for large network construction, BNs sacrifice network size for probabilistic robustness, making them more suitable for assessing central subnetworks and their dynamics within a larger gene network (Young et al., 2014). A BN was inferred for associations during sexual development and meiosis silencing in *N. crassa* using gene expression data, and knockout phenotypes showed high consistency between the order of the morphological development and tie structure of the predicted network (Wang et al., 2014). In the near future, genome-scale metabolic networks that are mechanistic in nature are expected to be able to be reconstructed with high precision and robustness (Al-Omari et al., 2015; Oguz et al., 2017). But for the level of complexity underlying evo-devo, both statistical networks such as Bayesian networks and molecular biological mechanistic networks are likely to continue to play roles for different levels of underlying knowledge and to complementarily reveal unknown network topology for developmental complexity.

The probabilistic formalism in Bayesian Network (BN) provides a robust and straightforward approach toward modeling the highly interactive components that are key to biological systems (Needham et al., 2007, 2006). However, to explore an unknown complex using Bayesian networks requires the development of the means for quantitatively describing and comparing networks—especially the dynamic changes in networks (Balaguer and Sozzani, 2017; Ghanmi et al., 2011; Li et al., 2011). In addition, quantitative and qualitative characteristics of

BN, including the probabilistic and mechanistic models, make it possible to precisely manipulate components of the network *in silico* before verifying them *in vitro*. Regarding investigation of genetic networks in fungal biology and development, a Maximally Informative Next Experiment (MINE), conceptually defined as the maximum distance (by some quantitative metric) between model-determined networks, provided predictions of the optimal perturbations to study the dynamics of a circadian network in *Neurospora crassa* (Dong et al., 2008). In this well-studied system, the action kinetics of circadian dynamics can be directly quantitatively measured, and the elegantly parameterized circadian network could be modeled with ordinary differential equations (ODE) representing the temporal profiles of genes and their products (Dong et al., 2008). Unlike the metabolic networks underlying circadian rhythms that can be quantified using established biochemical modeling approaches, developmental regulatory networks remain challenging to quantitatively describe using ODEs. The MINE concept, however, is general, and can be adapted to measure the robustness difference (posterior possibility space difference) of more statistical modeling approaches such as Bayesian networks, and thus be applied to predict the maximally informative next experiment.

3.2 Systems biology strategy to study fungal development using Bayesian network approach

Here, we develop the idea of systematically proposing experiments via robust Bayesian network ensemble modeling, with our focus on the identification of the maximally informative next experiment in fungal development. Generally, model parameters can be perturbed in accordance with experimental capabilities. The diversity of outcomes in response to each perturbation across the ensemble of individual models can be measured by the variance in predicted progression of development. The key idea is that the experiment with the highest diversity of predicted outcomes among best models is the one that will be most informative once the experimental result is known, as it will invalidate the greatest number of otherwise high-scoring models in the ensemble. The method above falls into the framework of active learning, and specifically corresponds to the querying strategy of uncertainty sampling to estimate the topology and parameters of a Bayesian network (Ness et al., 2017; Pauwels et al., 2014; Tong and Koller, 2001). At each step of the training process, the expected uncertainty of each experiment given the current posterior distribution of the graph parameters is calculated and the one with the highest expected uncertainty is chosen. Thus through several iterations of the systems approach we expect the posterior distribution over the model parameters to concentrate in a small subspace, ultimately narrowing down to one or a few strongly supported models to investigate with detailed molecular biological approaches.

This rapid narrowing of the plausible set of models down to one or a few that can be further investigated with gene-by-gene molecular biological techniques represents an optimal working strategy considering the supralinear complexity of a biological network with increasing numbers of network elements. Such an experimental process should combine iterative Bayesian network modeling, transcriptomics, and knockout phenotyping led by optimizing informativeness of genetic experiments on the developmental biology (Fig. 2). Genome-wide network

reconstruction approaches, such as GeCON using preliminary expression data collected and available prior knowledge from reference search and data mining can be used to identify a set of components (Roy et al., 2014) module extraction—algorithms for describing subnetwork space and modularity with edge density and strength—can then be used to identify a core set of interacting genes with a proper size (He et al., 2017). Three systems biological operations would then be expected to be implemented iteratively: 1) assembly of a number of BNs for the identified core network, 2) evaluation of the diversity of predictions from all putative experiments to infer a MINE, which would most successfully discriminate among models, and 3) performance of the experiment directed by a MINE criterion, and assessment of gene perturbation phenotypes. Next, one returns to step 1 again, taking newly generated data combined with previous data to construct a refined ensemble core network. These steps can be iterated until no significant information is obtained by conducting the predicted best experiment.

Such systems biology approaches can be further developed and applied at a lower level of fungal evo-devo to understand the evolution of the core regulatory complex during cell and tissue type differentiation (Arendt et al. 2016). Although most fungal structures are microscopic, as in *Neurospora crassa*, they are nevertheless composed of multiple cell and tissue types (Bistis et al., 2003). These microscopic structures have been challenging to examine until recently, with the development and application of laser dissection (Teichert et al. 2012) and single cell sequencing (Picelli 2016). Unlike macroscopic forms in morphological development, homology among cell and tissue types is often comparatively easier to establish, especially within closely related fungal species. Thus, the network of regulatory interactions governing their microscopic morphology will be of high interest to molecular geneticists and synthetic biologists.

3.3 Reconciling the evolutionary histories between the developmental networks and developmental traits

Developmental biology has been informed based on investigations using the tools of molecular biology and genetics, and also by the historical inference tools of evolutionary biology. A better understanding of systems biology in fungal developmental traits would be achieved with explicit reconciliation between functional/developmental networks and their evolutionary histories. In fact, species and gene phylogenies can reveal how certain developmental traits may have converged or diverged, and thus direct comparative genomics analyses that identify candidate genes underlying the development of traits. While it has been common for systems biological analyses of expression to inform networks of interest (Cracraft, 2005; Fu et al., 2017; Lu et al., 2014; Nowrousian, 2014; Teichert et al., 2014; Zhang et al., 2015), systems biological analyses examining multiple species and inferring historical patterns of gene expression can help to define the evolution of developmental networks of interest. Reconstruction of historical genetic networks along the species phylogeny could be especially helpful in illuminating genetic components of developmental traits that are regulated, often quantitatively or plastically, by multiple genes—whereas gene-by-gene phenotype analysis is only slowly, step by step,

informative for inferences regarding multiple gene-gene interactions (Li and Johnson, 2010; Shamir, 2008).

Comparative genomic data to identify orthologous genes, comparative transcriptomic data to reconstruct evolutionary changes of gene expression phenotypes across multiple species, and gene knockout technology in more than one species to interrogate function, can be pursued to bring comparative genetics, developmental biology, genomics, and transcriptomics into a single phylogenetic framework. In Trail et al. (2017), genetic networks during perithecial initiation and perithecial beak development were inferred using Bayesian network approach and compared among five Sordariomycetes models. Results showed that a core network of orthologous developmental processes is probably conserved in component and expression regulation among morphologically divergent species. Similarly, conserved involvement in mushroom development of transcriptional circuitry consisting of a few orthologous genes was suggested by a comparative transcriptomics on the model mushroom *Coprinopsis cinerea* (Plaza et al., 2014). Reconciliation among multiple species of the gene network and associated developmental traits—“syst-evo-devo” would directly bridge the hallowed terrains of morphological divergence and genetic/genomic/transcriptomic adaptation. This reconciliation is not just of disciplinary interest for fungal systematics and evolution, but also promises significant returns for investigators involved in other aspects of mycology—especially investigators working on fungal groups of special ecological and economic value. These fungi include many pathogens and industrial species that generally exhibit highly reduced morphology. Understanding the regulatory basis of the extant morphology and its relation to complex morphologies in model organisms would be critical for further work revealing key genes responsible for developmental transitions to stages of fungal life history that are of particular interest.

4. Concluding remarks on fungal development: past, present and future—reductive approaches to integrative systems biology strategies

Advances in quality and quantity from genomics and transcriptomics data, and simultaneous methodological advances associated with systems biology approaches, has the potential to lead to a new era for evolutionary developmental biology in diverse fungal species. Especially for most development phenotypes that are controlled by multiple factors modulated by a regulatory network, evolutionary systems biology provides a means to infer evolution of the regulatory network underlying developmental traits and to provide strategies to quantitatively and qualitatively verify functional roles of key components of the network. Developmental biology can now advance beyond a reductive molecular biological investigation that is heavily dependent on a pre-set understanding of development traits, which generally requires extensive trial experiments and complicated strategies of investigation that sacrifice statistical power for robustness of a few key findings. Recent advances in fungal genomics and transcriptomics greatly improve the efficiency of reductionist approaches by supplying focused targets for manipulations, yet investigation of regulatory networks for various developmental traits faces many challenges that are beyond the scope of gene-by-gene approaches. Integrating systems

biology into evo-devo can provide solutions to those challenges, since systems biology can rapidly incorporate the many components of the developmental process that are naturally in operation. Moreover, systems biology approaches can catalog key components of development into statistically optimal groups based on all available data from genetics, transcriptomics and evolutionary biology with large-scale computational intelligence, to significantly and rationally improve experimental strategies and efficiency. Gene regulatory networks incorporating time course expression data, comparative genomics, and species phylogeny make it possible to integrate reductive approaches and systems biology for understanding fungal development within an evolutionary frame. Dynamic model-based iterative experiment is a promising strategy toward revealing the genetic basis of fungal morphological development and its evolution.

“Nevertheless, genomics marks a shift away from the extreme reductionism of molecular biology, and allows insights into biological complexity never before possible. The whole is, indeed, greater than the sum of its parts.” (Bennett and Arnold 2001)

Declaration of interest

The authors declare no competing interests.

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Figure legends

Figure 1. Flow chart for ancestral ortholog predictions, RNAseq, phylogeny, and focused knockout phenotyping (modified from Trail et al., 2017).

Figure 2. Demonstration of Bayesian Network (BN) modeling strategy in the study of fungal development. A) Mined diverse sources of data to compose an initial model. B) Guide iterative perturbations and time-course experiments using a Maximally Informative Next Experiment (MINE) criterion (c.f. Dong et al., 2008). Bayesian scoring metrics can be defined with a graph G and data D . $P(G)$ denotes the prior distribution of the graph, $P(D|G)$ the likelihood function, and $P(G|D)$ the posterior distribution of the graph given the observed data D . At each iteration, BNs can be constructed to describe network function based on experimentally manipulable input functions of developmental stages and other non-genetic factors, as well as quantitative output of development traits. C) Design the next experiment, prioritizing those experiments predicted to yield diverse outcomes (morphological phenotypes and/or gene expression levels) based on the current ensemble of BNs. D) Iterate genetic and environmental perturbation experiments. A core of eight genes are depicted here with up- (white circles) or down-regulated (grey circles) expression. E) Infer dynamic edge associations within the Bayesian networks using time course data. Significant associations and covariances can be handled by using dynamic edge-network approaches.