Molecular Biology and Evolution, 2025, **42**, 1–11
https://doi.org/10.1093/molbev/msaf131
Advance access publication 2 June 2025
Discoveries

MBE OXFORD

Rates of Evolution of Developmental Changes in Gene Expression in Sordariomycetes

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Associate editor: Emily Josephs

Abstract

The phenotype of an organism is shaped by gene expression within developing tissues. This shaping relates the evolution of gene expression to phenotypic evolution, through divergence in gene expression and consequent phenotype. Rates of phenotypic evolution receive extensive attention. However, the degree to which divergence in the phenotype of gene expression is subject to heterogeneous rates of evolution across developmental stages has not previously been assessed. Here, we analyzed the evolution of the expression of single-copy orthologs within 9 species of Sordariomycetes Fungi, across 9 developmental stages within asexual spore germination and sexual reproduction. Rates of gene expression evolution exhibited high variation both within and among developmental stages. Furthermore, rates of gene expression evolution were correlated with nonsynonymous to synonymous substitution rates (dN/dS), suggesting that gene sequence evolution and expression evolution are indirectly or directly driven by common evolutionary forces. Functional pathway analyses demonstrate that rates of gene expression evolution are higher in labile pathways such as carbon metabolism, and lower in conserved pathways such as those involved in cell cycle and molecular signaling. Lastly, the expression of genes in the meiosis pathway evolved at a slower rate only across the stages where meiosis took place, suggesting that stage-specific low rates of expression evolution implicate high relevance of the genes to developmental operations occurring between those stages.

Keywords: gene expression, evolutionary genetics, development, Sordariomycetes

Introduction

Gene expression is a fundamental process in all organisms, bridging the gap between genetic material and functional biology. It is intricately regulated at multiple levels, including transcriptionally, post-transcriptionally, and proteomically, to promptly respond to biological, developmental, and environmental signals. Messenger RNAs—regulated transcriptionally and via RNA degradation—are the initial product of the hierarchy of gene activity regulation. Accordingly, RNA levels have been used as direct measures of gene activity in numerous genome-wide studies (Schwanhäusser et al. 2011; Liu et al. 2016; Buccitelli and Selbach 2020). RNA levels of genes can also determine the magnitude of selection pressure on amino-acid substitutions (Drummond and Wilke 2008; Park et al. 2013). Study of differentially expressed genes has enabled the assessment of the functional roles of genes (Ferreira de Carvalho et al. 2016; Gortikov et al. 2022), illuminated gene regulatory networks (Wang et al. 2014), and guided targeted experimental investigations (Kim et al. 2019). Temporally and spatially explicit investigations of gene expression have enabled the identification of genes involved in diverse biological and developmental functions (Mantri et al. 2021). However, genome-wide expression variation is present not only within an organism, but also has been documented among strains (Townsend et al. 2003; Glaser-Schmitt and Parsch 2023), species (Brawand et al. 2011; Israel et al. 2016; Gildor et al. 2019; Wang et al. 2020a), and specifically between paralogs (Gu et al. 2002). Patterns have emerged linking the divergence in gene expression to other biological metrics. For instance, divergence in expression is higher in testis (Brawand et al. 2011), higher in certain developmental stages (Israel et al. 2016; Wang et al. 2020a), and positively correlated with evolutionary distances (Jordan et al. 2005; Lemos et al. 2005; Gildor et al. 2019). Fundamentally, this diversity of gene expression level is driven by regulatory changes (McManus et al. 2010) and evolutionary forces of selection and drift (Lin et al. 2017), wherein selection on infrequent, high-effect, highly deleterious mutations typically maintains an average level of expression (Hodgins-Davis et al. 2015). Accordingly, modeling of gene expression as a stochastic process (Bedford and Hartl 2009; Rohlfs et al. 2014; Boucher and Démery 2016) enables the identification of divergent expression associated with divergent phenotypes or ecologies

Received: December 10, 2024. **Revised:** March 26, 2025. **Accepted:** May 2, 2025

(Munro et al. 2022; Liu et al. 2023) and the illumination of other evolutionary questions such as testing the developmental hourglass model (Kalinka et al. 2010) and the relation between neofunctionalization and gene duplications (Fukushima and Pollock 2020).

Just as discrete-state substitution rates are one of the most important parameters to estimate to characterize the evolution of divergent DNA and amino-acid sequences, continuous-state rates of evolution are one of the most important parameters to characterize the evolution of divergent gene expression. However, rates of evolutionary divergence in gene expression are not commonly reported—perhaps because estimating them meaningfully involves multiple challenges (Hodgins-Davis and Townsend 2009). Different approaches developed to address these challenges result in incommensurate metrics that are not suited to comparison. A particular challenge in multicellular organisms is the identification of homologous tissues to sample. The function of gene regulation, which is often to rapidly respond to the environment, gives rise to an even greater challenge: adequately controlling for environmental variables. Distinct environments—both earlier than (Smith and Kruglyak 2008) and highly proximate to (Sultan et al. 2014) the time of tissue sampling—substantially affect gene expression, yet many studies collected samples from diverse sources (Brawand et al. 2011; Munro et al. 2022; Liu et al. 2023). When possible, the cultivation of species of interest in a single condition along with reproducible sampling of specific developmental stages reduces these biases.

Once expression data is collected, another challenge regards the means for "normalization" of the expression data so that it has a common meaning for comparison across different species. A set of orthologs can have different gene lengths in each species. Consequently, well-understood sequence-read normalizations, reads per kilobase per million mapped reads (RPKM), transcripts per million or trimmed mean of M-values (TMM), account for expected numbers of sequenced fragments of a gene (Brawand et al. 2011; Catalán et al. 2019; Munro et al. 2022; Liu et al. 2023). However, even these normalized counts are often not directly comparable due to various factors (SEQC Consortium 2014). Using expression fold changes between different stages helps to abate these issues (Rifkin et al. 2003; Dunn et al. 2013; Trail et al. 2017), enabling meaningful measurements of the rates of evolution of the fundamental, evolvable trait of gene expression, and directing our attention toward genes whose expression evolves at corresponding paces with relevant biological traits.

Here, we aim to determine the spectrum of rates at which gene expression evolves and ascertain how those rates relate to protein sequence evolution and encoded functions in fungi. We hypothesize that (i) rates of expression evolution vary widely across the genome, (ii) gene expression evolves faster in sexual reproduction developmental stages, (iii) expression evolution is highly correlated with sequence evolution, and (iv) rates of expression evolution are divergent among genes with different functions and associated with developmental processes. Originating in the late Mesozoic era, ~230 million years ago, the class Sordariomycetes is a system that is well-suited for testing our hypotheses because of its phenotypic and morphological conservation in various developmental processes and the expansion into remarkable ecological diversity (Wang et al. 2023). These fungi produce small, flask-shaped, hazard-resistant perithecia, which bear meiotic spores (ascospores) during sexual reproduction, with well-defined developmental stages across species. Yet, perithecia result from different reproduction systems, such as heterothallism, homothallism, and pseudo-homothallism, while some members of Sordariomycetes do not have a known sexual component in their life history or are intensively involved in parasexual reproduction. Sordariomycetes also generate minute, single-celled mitotic spores (conidia) that rapidly germinate into hyphae and mycelia, facilitating swift propagation and colonization. Unlike the largely conserved perithecia, the diversity of conidia and their germination strategies contribute to the broad ecological range of the class. Members include saprotrophic species inhabiting soil and water, as well as parasitic species that infect a wide array of hosts, including plants, insects, mammals, and even other fungi.

We estimate the rates of gene expression evolution by quantifying the diffusion of gene expression changes between serial morphostages across the evolution of model species in the fungal class Sordariomycetes (Trail et al. 2017; Wang et al. 2023). In particular, we analyze sexual development across five model species, and asexual spore germination across six model species, computing the frequencies and times of halving or doubling of gene expression. We quantify these frequencies and times of halving or doubling of gene expression in alignment with the morphologies of sexual development and spore germination, which follow distinct yet homologous developmental progressions. Sexual development starts with the formation of hyphal nodes, progresses to their manifestation as protoperithecia, and continues with their intercrossing and growth of perithecia bearing asci that release sexual spores (ascospores). Similar phenotypically conserved morphologies of spore germination extend from isotropic expansion to polarized growth to elongation and hyphal branching. The conserved traits associated with the phenotypes of asexual spore germination and sexual development enable quantification of rates of change of gene expression linked to key morphological developmental transitions, and facilitate comparison of the molecular evolution of gene expression to molecular evolutionary sequence divergence in individual genes as well as pathways associated with the development of serial morphological phenotypes.

Materials and Methods

Gene Expression Dataset

Two datasets composed of genome-wide gene expression were used to analyze the rate of gene expression evolution (supplementary table S1, Supplementary Material online). The dataset on sexual development was collected from 5 model fungi in the Sordariomycetes: *Neurospora crassa*, *Fusarium graminearum*, *Fusarium neocosmosporiellum*, *Magnaporthe oryzae*, and *Chaetomium globosum* at 5 distinct morphological stages that are conserved within the species, mature protoperthecia, fertilized perithecia, development of an ascogenous center, the appearance of asci and ascospores, and the release of mature ascospores, during their sexual reproduction (Fig. 1; Trail et al. 2017). The individual fungi studied here exhibit fairly synchronous morphological development when cultured on carrot media (Wang et al. 2012; Lehr et al. 2014; Trail et al. 2017), but they take different times to reach these distinctive morphological transitions (supplementary table S2, Supplementary Material online). The second dataset on asexual spore germination was

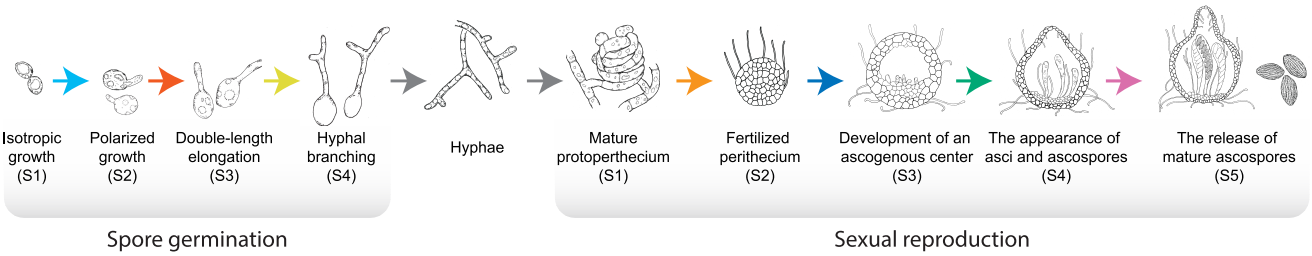

Fig. 1. Illustrations of morphologically defined developmental stages of asexual spore germination, hyphal mat formation, protoperithecia formation, and sexual development across the fungal life cycle of Sordariomycetes.

collected from 6 model fungi in the Sordariomycetes, *N. crassa*, *F. graminearum*, *Trichoderma asperelloides*, *Tolypocladium ophioglossoides*, *Cordyceps militaris*, and *Metarhizium anisopliae*, representing saprotrophs, phytopathogens, entomopathogens, or mycoparasites, which were sampled at 4 morphological stages, isotropic growth, polarized growth, double-length elongation, and hyphal branching, during asexual spore germination on a common potato dextrose agar (PDA) medium (Fig. 1). As with sexual reproduction, expression dynamics across these 4 stages of spore germination are comparable among divergent ascomycetes species (supplementary table S2, Supplementary Material online; Wang et al. 2019; Miguel-Rojas et al. 2023; Kim et al. 2025). RNA sequencing data for *N. crassa*, *F. graminearum*, and *Tr. asperelloides* were collected as part of previous analyses of genome-wide gene expression regulation during conidia germination in response to different environmental settings (Gortikov et al. 2022; Wang et al. 2022; Moonjely and Trail 2024). RNA sequencing data for the other 3 species (NCBI GEO: *Co. militaris* and *M. anisopliae*: PRJNA1171587; *To. ophioglossoides*: PRJNA1177519) were collected for this study following meticulously identical methodology as in Wang et al. (2022) and in Trail et al. (2017), described in the following sections. Relative expression values were estimated stage by stage with LOX (Zhang et al. 2010) and expression changes between adjacent stages were calculated as $\frac{x_{s+1}-x_s}{\min(x_s, x_{s+1})}$, where x is the relative expression level and s indicates the stage. LOX is especially well-suited to inference of the levels of expression of each gene across samples on a scale normed to the gene's expression in the lowest-expressed sample. Having results in this form keeps data close to its natural distribution, minimizing the number of statistical transformations from the raw data to conduct the difference-of-differences analysis. To enable gene-specific estimation of gene expression among different species, single-copy orthologs were identified with ReMark and BranchClust (Trail et al. 2017; Kim et al. 2022). To estimate the absolute expression level of each gene as indicated by sequence-read counts, TMM-RPKM values for *N. crassa* genes without alternative transcripts were calculated with R package *edgeR*.

Culture Conditions

Cordyceps militaris strain CGMCC 3.16322 and *Metarhizium anisopliae* strain ARSEF 23 were used in the conidia germination studies. The conidia were harvested with deionized distilled water containing Tween 20 (0.1%) from 20-day cultures for *C. militaris* and 15-day cultures for *M. anisopliae* on PDA medium. They were washed twice with autoclaved distilled water and filtered through a 3-layer Mira cloth to gather spores without hyphae. The 10^5 conidia in solution were placed on top of cellophane-covered PDA medium to investigate the program of germination. *Co. militaris* conidia were incubated under constant dark at 20 °C, while *Me. anisopliae* conidia were at 25 °C. Germination was monitored at 0, 5, 10, 15, 18.5, 20, 23, 25, 30, 35, 40, 43, and 45 h. The tissues of *C. militaris* with cellophane membranes were collected at 0, 15, 18.5, and 43 h, when the majority of active spores on PDA were at one of the following stages and beyond: fresh spores, spores showing evidence of polar growth, spores having doubled their long axis, and spores having first hyphal branching. The tissues of *Me. anisopliae* were collected at 0, 5, 10, and 23 h for 4 distinctive morphological stages of germination. Tissue samples were flash-frozen in liquid nitrogen and stored at −80 °C. All tissues that were collected from multiple plates in 1 collection process were counted as 1 biological replicate. Three temporally segregated biological replicates were prepared for each stage sample.

RNA Isolation and Preparation

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA quality and concentration were monitored on agarose gels and a Nanophotometer (Thermo Scientific, Thermo Fisher Scientific Inc., Asheville, USA). RNA integrity was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA). Twenty-four libraries (2 strains × 4 stages × 3 biological replications) were generated using VAHTS mRNA-seq v2 Library Prep Kit for Illumina (Vazyme Biotech Co., Ltd, Beijing, China) following the manufacturer's instructions.

Transcriptome Sequencing and Quality Control

The cDNA libraries were sequenced on an Illumina NovaSeq platform (Illumina Inc., USA) by Berry Genomics Co. (Beijing, China) to generate 150-bp paired-end reads according to the manufacturer's instructions. Clean data were obtained by removing sequences containing adapters, poly-Ns, and low-quality reads from the raw data. Trimmed reads were aligned to the genomes from the Broad Institute using HISAT2 v2.1, indicating that reads correspond to the reverse complement of the transcripts and reporting alignments tailored for transcript assemblers. Alignments with a quality score below 20 were excluded from further analysis. Reads were counted for each gene with StringTie v1.3.3 and the Python script prepDE.py provided in the package. StringTie was limited to report reads that matched the reference annotation.

Evolutionary Modeling of Gene Expression

A timed phylogeny inferred from genomes of 1644 fungal species (Li et al. 2021) was calibrated to a 230 MYA age for the

most recent common ancestor of the 9 studied species (Berbee and Taylor 2010). Using the R package *geiger* and specifying Brownian motion and Ornstein–Uhlenbeck models along this timed phylogeny, we fit the expression changes between the serial stage pairs. For each gene, we extracted their diffusion parameter and calculated the rate of gene expression evolution as the frequencies of halving or doubling of the change in gene expression in a million years.

Substitution Rate and dN/dS

To measure the substitution rate for each gene, the protein sequences of each orthologous group were aligned with MAFFT ver. 7.505. The protein alignments were trimmed with TrimAl ver. 1.2rev59, and phylogenies were reconstructed using IQ-TREE2 ver. 2.1.2 specifying the MFP model and the species phylogeny as a constraint (Minh et al. 2020). To estimate amino-acid substitution rates, the sums of branch lengths of the gene trees were divided by the sum of the branch lengths of the species tree. To infer dN and dS, annotated coding sequences were aligned to the protein alignments. Maximum-likelihood ancestral sequences were reconstructed via the approach implemented with Single-Likelihood Ancestor Counting in HyPhy ver. 2.5.48, which also quantified nonsynonymous and synonymous changes using a modified version of the Suzuki–Gojobori method (Kosakovsky Pond and Frost 2005).

Functional Analyses

Gene-set enrichment analyses were performed to identify the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways that were associated with faster or slower rates of gene expression evolution. Specifically, we used the gseKEGG function from the R package *clusterProfiler* to estimate parametric enrichment scores of evolutionary rates (Wu et al. 2021).

Statistical Analyses

To identify the best-fit distributions of the evolutionary rates of gene expression without being affected by outliers, we disregarded the first and last 10 percentiles of data (van der Loo 2010). To characterize the best analytical form to describe this rate distribution, we fit the remainder to normal, log-normal, exponential, Pareto, and Weibull distributions, using the R package *extremevalues*. All correlation tests were conducted using Spearman's correlation. Partial correlations were performed with the R package *ppcor*. Kruskal–Wallis tests were used to compare among multiple groups and were followed by Dunn's test.

Results

A Wide Spectrum of the Evolutionary Rate of Gene Expression

Among the 3,941 single-copied orthologs, the lower quartiles of the rates of gene expression evolution (the frequencies of halving or doubling of change in gene expression in million years) between serial stages of sexual reproduction ranged from 4.0×10^{-4} to 9.2×10^{-4} million year^{-1}, and the upper quartiles of the rates of gene expression evolution between serial stages of sexual reproduction ranged from 2.8×10^{-3} to 8.4×10^{-3} million year^{-1}. The distribution of these evolutionary rates was roughly log-normal ($r^2=0.93$ to 0.99), with a positive skew (35 to 66 outliers; Fig. 2a, b, d, and e; supplementary tables S3 and S4, Supplementary Material online). By taking half of the inversion of the rate, the time required could be estimated for a gene to reach a variance in expression change of 1 between stages (equivalent to doubling or halving expression change on average; Fig. 2a, b, d, and e; supplementary table S3, Supplementary Material online). The median times required for genes to reach a variance in expression change of one ranged from 405 to 966 million years across the 4 serial stage pairs. The rates of gene expression evolution were positively correlated between serial stage pairs ($P<0.001$). However, the correlation coefficients of rates of evolution between serial stage pairs were moderate, ranging between 0.34 and 0.50 (supplementary fig. S1, Supplementary Material online).

The rates of evolution of the change in gene expression occurring from polarized growth to double-length elongation and from double-length elongation to the first hyphal branch were similar to the rates of evolution from morphostage to morphostage during sexual reproduction. Among the expression rates of 4,017 genes, the lower quartiles were 7.5×10^{-4} and 6.3×10^{-4} million year^{-1}, and the upper quartiles were 5.3×10^{-3} and 5.6×10^{-3} million year^{-1} (Fig. 2a, c, d, and f; supplementary tables S3 and S5, Supplementary Material online). Accordingly, the median times required for genes to reach a variance in expression change of one between stages were 539 million years and 597 million years, respectively (Fig. 2a, c, d, and f; supplementary table S3, Supplementary Material online). However, gene expression during the transition between isotropic growth (stage 1) and polarized growth (stage 2) of asexual spore germination evolved at a much higher rate. The span of the lower and higher quartiles was 4.3×10^{-2} to 6.1×10^{-2} million year^{-1}, and the median time required for genes to reach a variance in expression change of one was 6.9 million years. The Spearman's ρ correlation coefficients of rates of evolution between serial stage pairs during asexual spore germination ranged from 0.22 to 0.30: lower than those observed across stages of sexual development (supplementary fig. S2, Supplementary Material online).

The Rates of Gene Expression Evolution Correlate Positively With Substitution Rates and dN/dS

Because gene expression is related to gene function, some correlation between the evolutionary rate of the continuous trait of gene expression and the evolutionary rate of the discrete trait of gene sequence could be expected. Indeed, amino-acid substitution rates in a gene were positively correlated with the rates of gene expression evolution between every serial pair of stages of sexual reproduction ($P<10^{-4}$; $\rho=0.064$ to 0.205; Fig. 3a–d) and of asexual spore germination ($P<10^{-4}$; $\rho=0.068$ to 0.074; Fig. 3e and f). To understand if this positive correlation resulted from elevated mutation rates or from selection pressure, we tested the correlation of nonsynonymous divergence (dN), synonymous divergence (dS), and their ratio (dN/dS) with the rates of gene expression evolution. The dS—generally associated with underlying mutation rates and divergence times rather than the action of natural selection—was weakly negatively to weakly positively correlated with the rates of gene expression evolution between every serial pair of stages of sexual reproduction ($P<0.05$ for all comparisons; $\rho=-0.086$ to +0.059) and insignificantly to weakly positively correlated with those of asexual spore germination ($P>0.05$ for first and second stage pair and third and fourth stage pair; $P=0.0008$ for second and third stage

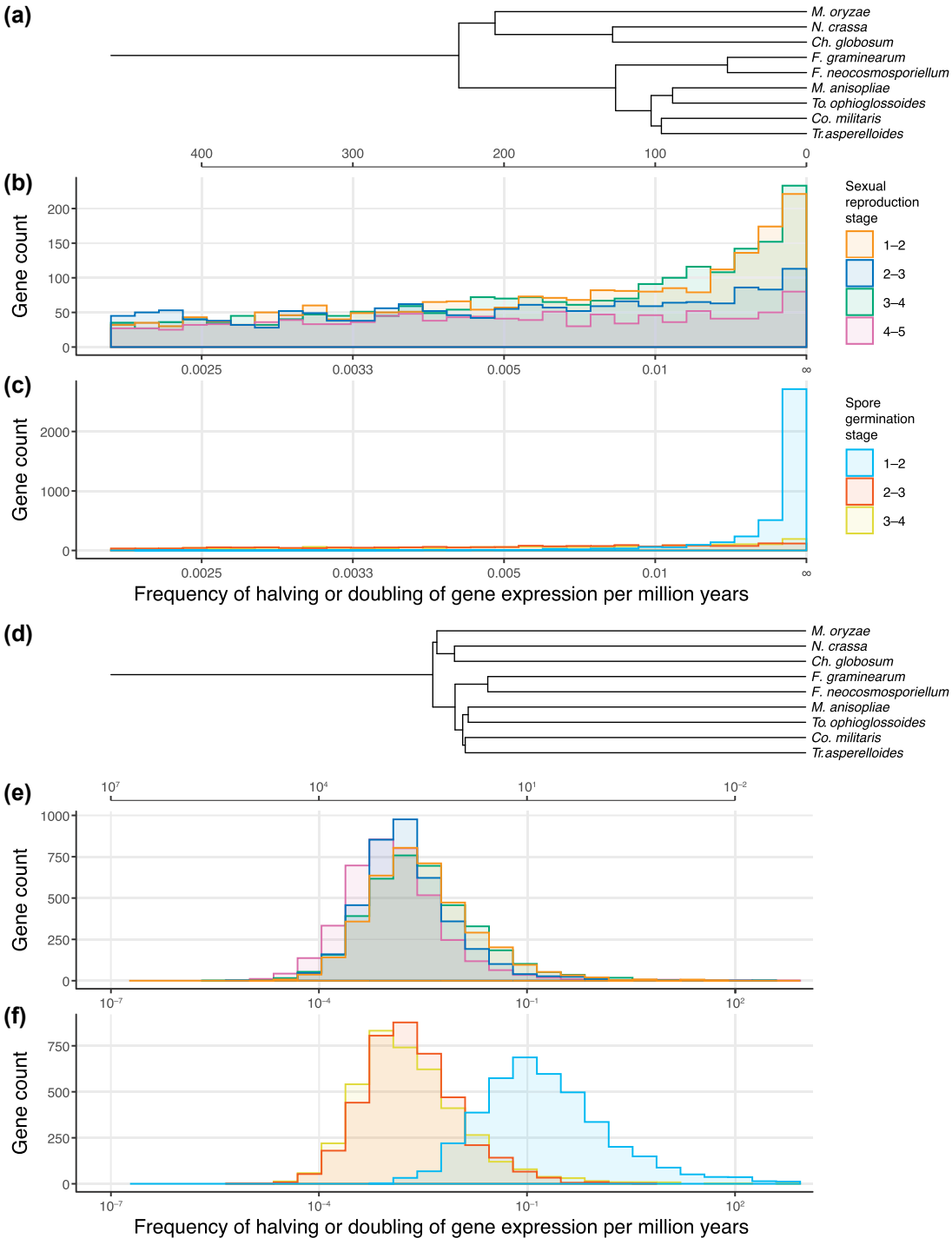

Fig. 2. Rate of gene expression evolution: (a) untransformed time tree depicting the phylogenetic relationships among the species; untransformed time required to evolve to achieve a variance of one in expression (top axis) and its frequency (bottom axis) between serial stages of (b) sexual development (c) asexual spore germination; (d) $\log_{10}$-transformed time tree depicting the phylogenetic relationships among the species; $\log_{10}$-transformed time required to evolve to achieve a variance of one in expression (top axis) and its frequency (bottom axis) between serial stages of (e) sexual development and (f) asexual spore germination.

pair; $\rho = 0.001$ to 0.054). The absolute correlation of dS with rates of gene expression evolution between most serial pairs of stages was substantially weaker than the positive correlation with the amino-acid substitution rate. Indeed, the correlation of dN with the rates of gene expression evolution between every serial pair of stages was comparable to the correlation of amino-acid substitution rates with the rates of gene expression evolution between every serial pair of stages ($P < 10^{-5}$; $\rho = 0.075$ to 0.200), as was dN/dS ($P < 10^{-3}$; $\rho = 0.058$ to 0.201; Fig. 3). Because previous studies have demonstrated a correlation between gene expression level and the rates of amino-acid substitution (Pál et al. 2001; Larracuente et al. 2008), we performed a partial correlation analysis between the rates of gene expression evolution and dN, controlling for the effects of dS and average gene expression level. Consistent with the results above, the rates of gene expression

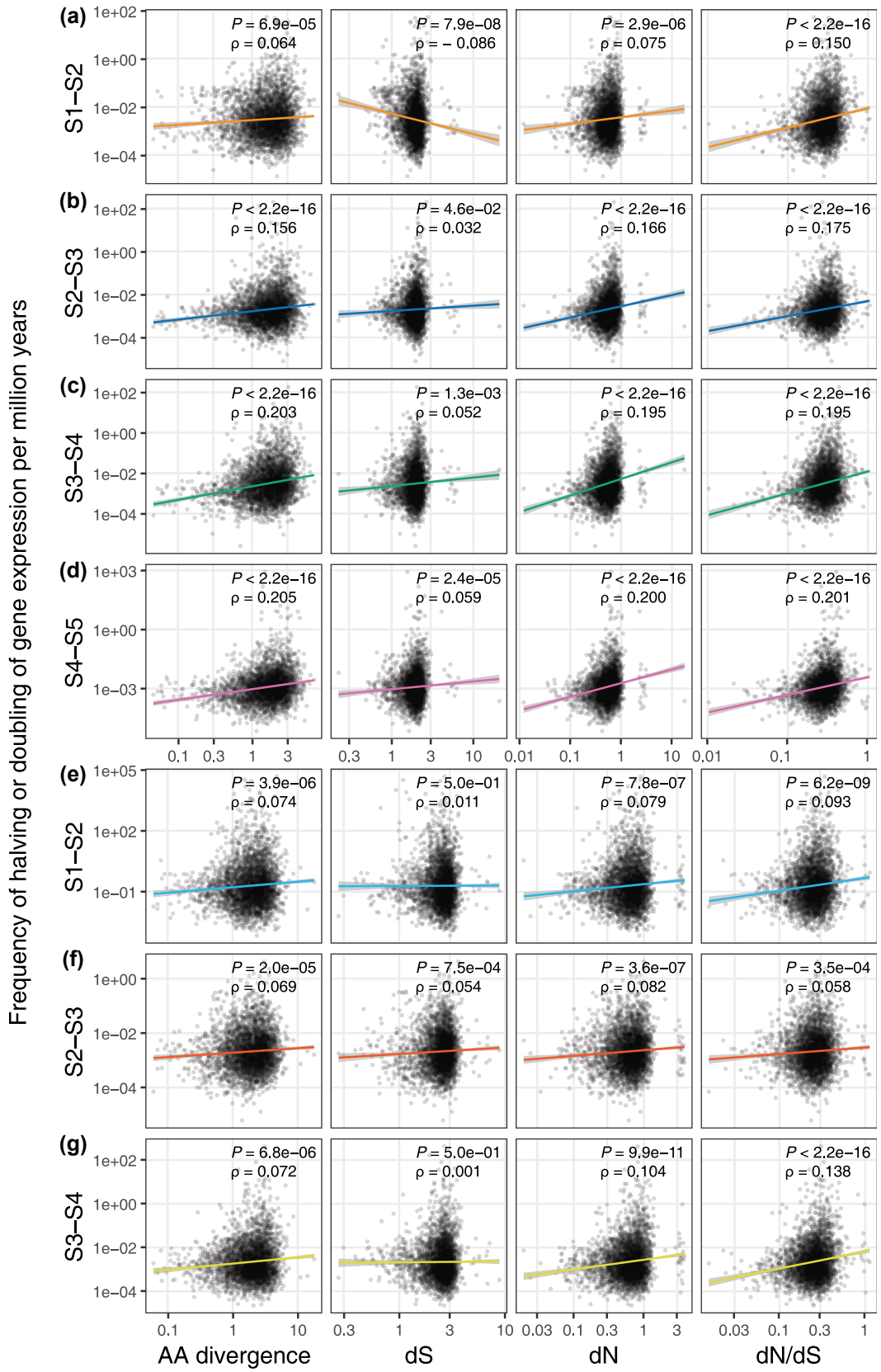

Fig. 3. Correlations between rates of gene expression evolution and total amino-acid divergence (substitutions), synonymous-site divergence (dS), and nonsynonymous-site divergence (dN), and their ratio dN/dS across 7 transitions between stages of (a) sexual development from mature protoperithecia (S1) to fertilized perithecia (S2), (b) to development of an ascogenous center (S3), (c) to the appearance of asci and ascospores (S4), (d) to the release of mature ascospores (S5); and (e) asexual spore germination from isotropic growth (S1) to polarized growth (S2), (f) to double-length elongation (S3), and (g) to the first hyphal branch (S4).

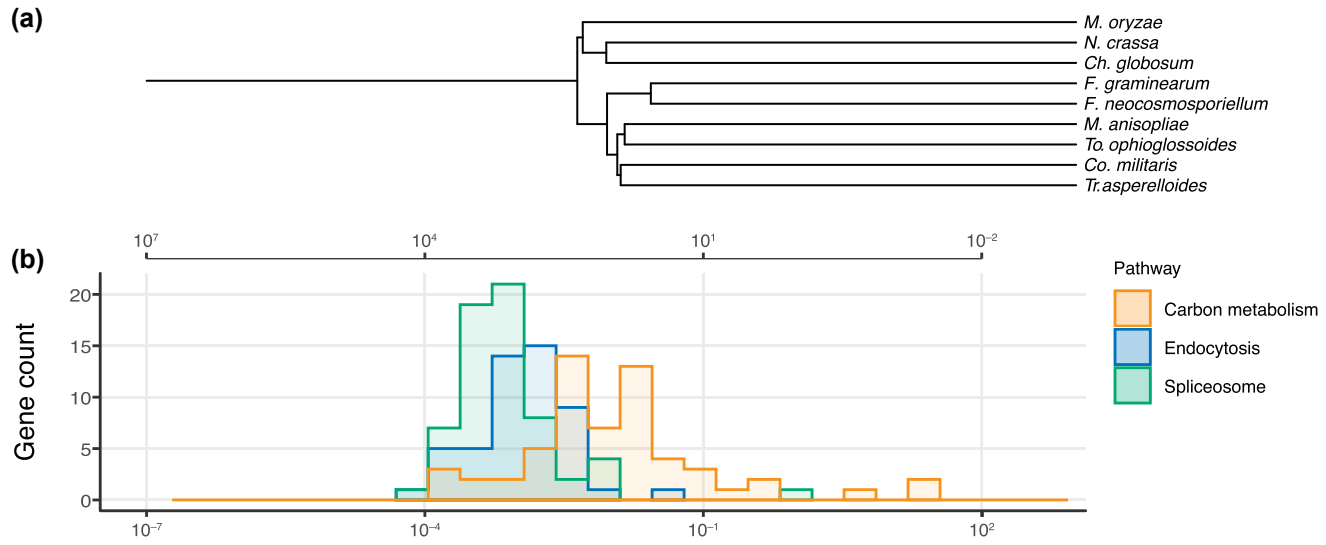

Fig. 4. Evolution of expression in genes within selected KEGG pathways at the early sexual reproduction stage: (a) phylogenetic relationship among the species; (b) density plot across genes for the time that would be required to evolve a variance of one (top axis) and its frequency (bottom axis) in the probability distribution of expression. Faster evolutionary rates (shorter times to evolve) were enriched within carbon-metabolism genes. Slower evolutionary rates (longer times to evolve) were enriched within genes of the spliceosome. Neither faster nor slower rates of evolution were enriched within genes whose function has been associated with endocytosis (for example). The *x* axis is log-transformed: within and among gene sets, there was a high variance in the rates of gene expression evolution.

evolution exhibit a strong correlation with dN of genes between every serial pair of stages in sexual reproduction ($P < 10^{-15}$; $\rho = 0.150$ to 0.201) and in asexual spore germination ($P < 10^{-3}$; 0.058 to 0.138).

Expression of Metabolic Pathways Evolves Faster

Gene-sets associated with phenotypes that are highly conserved were expected to exhibit low levels of functional change in phenotype and genotype, and accordingly less evolution of gene expression during the life cycle. Gene sets associated with phenotypes that were evolutionarily labile were expected to exhibit greater functional change in phenotype and genotype, and accordingly greater evolution of gene expression during the life cycle. To test if any gene sets evolve faster or slower than most genes, we employed enrichment analysis for the KEGG terms of orthologous gene sets. At the earliest 2 stages of sexual development when the perithecial wall differentiated, genes within a number of metabolic pathways exhibited enrichment for higher rates of gene expression evolution, such as genes in pathways associated with carbon, sulfur, methane, phenylalanine, tyrosine, and tryptophan metabolism. In contrast, gene sets associated with genetic information processing and cellular processes were enriched for slower rates of evolution, including gene sets composing the spliceosome and proteasome, as well as gene sets functioning in nucleocytoplasmic transport, chromatin remodeling, mitophagy, and autophagy (Fig. 4; supplementary fig. S3a, Supplementary Material online).

Similar associations manifested between the second and third stages of sexual development (spanning the development of paraphyses) and between the third and fourth stages (encompassing the development of asci from the ascogonia, but not ascospore differentiation (supplementary fig. S3b and c, Supplementary Material online). In the last 2 stages profiled (during which meiotic products, ascospores, become delimited, developing thick cell walls and readying for release), no gene sets exhibited statistically significantly higher rates of evolution. However, similar gene sets continued to be enriched for significantly slowly evolving gene expression (supplementary fig. S3d, Supplementary Material online). Notably, genes associated with meiosis exhibited a marked enrichment for slow evolutionary rates of gene expression exclusively between the third and fourth stages, during which meiosis occurs.

During spore germination, as in sexual reproduction, the genes within certain metabolic pathways were enriched for faster evolution of gene expression in each stage pair (supplementary fig. S4, Supplementary Material online). These pathways included starch and sucrose degradation, fructose and mannose degradation, and pentose and glucuronate interconversions, as well as valine, leucine and isoleucine degradation and glycerolipid metabolism. In contrast, limited pathways were enriched with genes whose expression evolved at slower evolutionary rates, such as with mRNA surveillance pathway and nucleocytoplasmic transport. Interestingly, gene sets associated with the proteasome were enriched for higher evolutionary rates extending from isotropic expansion of the spores to polarized growth. In contrast, the same gene sets were enriched for lower rates extending from hyphal elongation to hyphal branching. These differential evolutionary rates are consistent with functional divergence in the initial stages as well as involvement in critical conserved functions in later stages of spore germination.

We further investigated whether the rates of gene expression evolution correlate with the rates of sequence evolution within specific KEGG pathways. We studied 3 pathways that were not enriched for faster or slower rates, were enriched for faster rates, and were enriched for slower rates of gene expression evolution at the earliest 2 stages of sexual development, namely endocytosis, carbon metabolism, and spliceosome. The carbon-metabolism gene set has significantly higher dN/dS ($P = 6.1 \times 10^{-3}$, post-hoc adjusted $P < 0.05$; supplementary fig. S5, Supplementary Material online), supporting the hypothesis of an association between gene expression evolution and sequence evolution.

Rate Estimates From Brownian Motion and Ornstein–Uhlenbeck Process Models are Similar

To examine the robustness of our findings to the model of continuous trait evolution, we explored how diffusion parameters differed under Brownian motion and Ornstein–Uhlenbeck models. Like Brownian motion, the Ornstein–Uhlenbeck process is stochastic and often used for modeling continuous traits. However, the Ornstein–Uhlenbeck process includes an additional stabilizing selection parameter α that applies across all species during the time spanned by the phylogeny. In all 7 serial stage pairs, Brownian motion was always deemed more suitable than the Ornstein–Uhlenbeck process, based on an analysis of the corrected Akaike information criterion (AICc). Additionally, under both sexual development and spore germination, diffusion parameters under Brownian motion and Ornstein–Uhlenbeck process were significantly positively correlated ($P < 2.2 \times 10^{-16}$; Spearman's $\rho = 0.560$ to 0.737; supplementary fig. S6, Supplementary Material online). The selection parameter α was positively correlated with the diffusion parameter ($P < 2.2 \times 10^{-16}$; Spearman's $\rho = 0.550$ to 0.874; supplementary fig. S7, Supplementary Material online).

Discussion

Our analysis of gene expression evolution revealed a wide spectrum of rates of gene expression evolution across developmental morphostages of asexual spore germination and sexual development. Among 9 Sordariomycetes with a common ancestor some 230 million years ago, the estimated average time needed for developmental stage-to-stage changes in gene expression to double or halve from their current levels varied both among genes within developmental stage transitions and among developmental stage transitions. In most of the developmental transitions of morphological state, the lower quartiles of these times-of-evolution are ~200 million years, the median times-of-evolution are ~600 million years, and the upper quartiles are ~2 billion years. This degree of variation from gene to gene is indicative of both extremely strong purifying selection on gene expression (in the case of genes that remain unchanging from stage to stage over extremely long time spans), and extreme lability of gene expression (in the case of genes whose expression evolves from ancestor to descendent on much shorter time scales). Intriguingly, these times-of-evolution were significantly lower for gene expression changes occurring between isometric to polarized growth in asexual spore germination, where the median time of evolution was only 6.9 million years. These faster rates suggest greater expression divergence at early developmental stages that may link to the labile heterotrophic ecology of fungal species.

Furthermore, the rates of gene expression evolution were uniformly positively correlated with amino-acid and nonsynonymous substitution rates, as well as dN/dS, consistent with reports based on divergence between 2 species (Jordan et al. 2005; Lemos et al. 2005). Among KEGG pathways associated with faster and slower rates of evolution, we found an association between meiosis and slower expression evolutionary rates between the stages bookending meiosis. This association suggests that low rates of gene expression evolution are associated with the highly conserved functions involved in the intricate and precision timing of activity of meiotic genes that were active between these 2 developmental stages. Conversely, during spore germination, the expression of many metabolic pathways was found to vary significantly across the sampled species, potentially reflecting the key role of spore germination in their specific lifestyles. For example, germinating spores of the post-forest fire fungal model *N. crassa* can rapidly metabolize diverse carbohydrates such as cellulose and starch, which are abundantly supplied by moribund vegetation at burn sites; spores of the entomopathogenic fungus *Co. militaris* and *Me. anisopliae* require successful targeting to invade and degrade insects equipped with chitinous exoskeletons and fat-rich integuments; and spores of plant pathogens like *F. graminearum* must rapidly overcome the specific and effective defenses of plant epiphytic structures.

Lineage-specific expression patterns have likely driven such traits. An examination of knockout of genes with strong divergence in expression in *N. crassa* and *F. graminearum* during sexual reproduction has identified more mutants than systematic, whole-genome knockout approaches (Trail et al. 2017). Further studies on expression patterns associated with certain traits can help unveil their underlying molecular mechanisms.

Faster Gene Expression Evolution During Early Spore Germination May be a Consequence of Developmental Dynamics in Accordance With Ecological Roles

Genes exhibited a diversity of rates of expression evolution across the 7 pairs of morphologically defined developmental stages, with a moderate correlation in the rates of evolution between serial pairs of stages. Most intriguing was the markedly higher rates of gene expression evolution during the span from isotropic growth of asexual spores to their polarized growth. One explanation is that the nutrition-seeking modes of early stages of spore germination are crucial, and often in a species- or genus-specific way, to the lifestyle of each fungus, so that gene expression in stages of early spore germination may change rapidly when compared across the fungal kingdom. For example, within a single genus, the size of a spore can shape the dispersal in the atmosphere and therefore the survival of the fungus associated with its ecology and pathobiology (Golan et al. 2023). These rapid divergences in ecology may have resulted in the rapid evolution of gene expression in these stages, analogous to higher gene expression divergence that has been observed within a single species in the early stages of the development of annelids (Harry and Zakas 2024). Alternatively, the discrepancy can be attributed to the decisions to sample developmental stages for this research so as to provide visual clarity of definition and homology across divergent species. During early cellular growth in *Saccharomyces cerevisiae*, gene expression has been demonstrated to change very rapidly and highly dynamically (Geijer et al. 2012). Rapid, dynamic changes across morphostage transitions within species would lead to larger changes between species. In other words, the high rates of gene expression evolution observed in these stages in our study may correspond to our visual morphological staging, inadvertently leading to the capture of multiple early developmental stages. We addressed this challenge by aligning comparable developmental stages based on key morphological features that have distinct times of appearance, rather than relying on rigidly determined chronological time points that may have little connection to developmental biology (supplementary table S2, Supplementary Material online). A more fine-scaled sampling across these early stages of asexual spore germination could enable the identification of morphologically similar but

molecularly distinct stages and slower rates of gene expression evolution.

Brownian Motion Sufficiently Models Gene Expression Evolution, but does not Necessarily Describe How it Evolves Over All Time Scales

Several stochastic processes have been proposed to describe the evolution of continuous traits. In our project, we used Brownian motion processes, in which a trait undergoes random fluctuations throughout evolutionary history. Under an Ornstein–Uhlenbeck process, these fluctuations are modified to include a tendency for traits to fluctuate to an optimal value, matching the expectation for long-term stabilizing selection operating consistently across all modeled species (Hansen 1997; Bedford and Hartl 2009). Our comparison of the fit of the data to Brownian motion and Ornstein–Uhlenbeck processes identified moderately positive correlations between the diffusion parameters between the 2 models. Brownian motion models exhibited a better fit as quantified by corrected AICc. The better fit to Brownian models, however, does not indicate that gene expression is under neutral evolution, for several reasons. First, the experimental design, featuring the high complexity of culturing multiple species and sampling at specific developmental time points for global gene expression analysis, leads to a high sample size in terms of genes assayed, but a relatively small number of species in our phylogeny, which decreases statistical power to discern better fits for higher-parameter models. Moreover, the conception that any single evolutionary regime has been maintained across a time tree spanning hundreds of millions of years is fairly naive: Brownian motion is one model that could be expected to fit the data if many different species-specific regimes of selection were in serial operation over the elapsed period. No simple model likely would fit well with the actual evolutionary history if genome-wide gene expression levels under these conditions could be measured continuously over an era. Despite this model complexity mismatch, the diffusion parameter of a Brownian motion process manifestly captures the overall rate of gene expression evolution on this timescale as driven by multiple constraints, including mutation rate, regulatory architecture, selection pressure, and other factors (Moses and Landry 2010; Gildor and Smadar 2018; Fuqua et al. 2020).

Multigene Families May Require More Complex Models of Evolution

Our analysis only quantified the rates of evolution of single-copy genes rather than genes belonging to gene families. Genes whose families expand within a species phylogeny represent an example where the mismatch with a continuous model is well-known: new paralogs can be subject to relaxed selection pressure and neofunctionalization (Assis and Bachtrog 2013; Escorcia-Rodríguez et al. 2022), each of which would presumably affect the rates of gene expression evolution. The evolution of expression of such gene families might be consistent with complex models such as Ornstein–Uhlenbeck processes with multiple selective regimes—a hypothesis that has been explored in some animal systems (Fukushima and Pollock 2020; Munro et al. 2022). Indeed, estimation of the rates in complex gene families could enable a more holistic view of gene expression evolution. However, we did not attempt to estimate the evolutionary rates of these families: such an analysis would require well-resolved and dated gene trees made difficult by incomplete lineage sorting, duplications and losses, heterotachy, and numerous horizontal gene transfers in fungi (Fitzpatrick 2012; Wang et al. 2020b; Steenwyk et al. 2023).

Conclusion

By assessing highly conserved and critical developmental processes associated with simple hyphal morphology and the complex production of meiotic spores via carefully controlled experimental designs, we have determined that individual gene expression evolves at a phenomenally wide span of rates. These rates are correlated with gene function, and conserved expression patterns of genes are typically associated with the conservation of amino-acid sequence in proteins and perhaps with their functional relevance to the developmental stages. Consistent with a fundamental role in the evolutionary process, the rates of gene expression evolution have important relationships with organismal phenotypes. Quantification of the rates at which gene expression evolves is a crucial step toward understanding how gene expression change underlies organismal evolution. This step anticipates a generalization to both complex gene networks and complex gene families that would in turn deepen understanding of organismal evolution.

Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online.

Acknowledgments

We thank the support from the HPC facilities operated by the Yale Center for Research Computing.

Funding

This study was funded by grant numbers IOS 1916137 and MCB 2300123 from the U.S. National Science Foundation (Y.W., Z.W., and J.P.T.) and 5R01AI146584-04 from the U.S. National Institutes of Health (J.P.T.).

Data Availability

The newly generated transcriptome is publicly available in the Gene Expression Omnibus (GEO) under Bioproject numbers PRJNA1171587 and PRJNA1177519.

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