

Coordinated gene family evolution shapes the genome of dimorphic Mucorales

Corresponding Author: Dr Francisco Nicolás

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript thoroughly investigates 2 paralogous gene clusters that appear to be responsible for dimorphism in Mucorales. The authors then identified 2 novel regulatory genes that are necessary for yeast conversion. Finally, they complete comparative genomics and show that the presence/absence of the genes is potentially diagnostic for yeast conversion among Mucorales.

This is an intriguing hypothesis and the data presented support the conclusions. This will be of great significance to the field and potentially highly significant if this pattern holds true for the thermal dimorphs in general.

It appears that the analyses are complete, and all needed raw data are available (GEO submission and GitHub). I did not reanalyze any data, but the analyses use standard methods that should be able to be replicated, as sufficient detail is given in the methods.

Some minor comments:

Title should make it clearer that this is about Mucor, same for the abstract. It isn't until line 62 that Mucorales is mentioned. The comment is mainly because there are many dimorphic fungi and this could lead the reader to infer broader application. Morphological switching is common for fungi, and not just related to yeast/hyphae but many morphologies: sexual structures, mushroom formation, chlamydospores, storage structures, etc. So, be specific in the yeast/hyphae morphology addressed but this study in the introduction, while alluding to how this could apply to diverse morphologies in the discussion

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)

In the manuscript by Tahiri et al., the authors investigated the molecular basis of dimorphism in the human pathogenic fungus *Mucor lusitanicus*, as well as the conservation of gene families and expression patterns in *Mucor* and *Rhizopus* species within the Mucorales order. Dimorphism in fungi, especially in human pathogenic fungi, is crucial for the transition from an environmental form to a parasitic (host) form. Thus, this study addresses an important question in the fungal field. However, the manuscript offers a moderate conceptual advancement in the field of fungal dimorphism. The experimental approach includes transcriptomics performed with *M. lusitanicus* grown to different developmental stages (yeast-like, primary mycelium, reverse transition, mature mycelium), gene deletions, and characterization of their effect on transcriptome changes, and pull-down assays using DNA probes. Main concerns are the frequent overinterpretation of results, and the lack of experimental details. The transcriptome results and the generated mutants can be useful to the Mucorales researchers, but the scope remains limited.

Below are some major points that can be considered to improve the manuscript:

-Abstract/background: The concept of dimorphism has been overemphasized without the description of many dimorphic fungi. The many members of the Onygenales order, including *Coccidioides*, *Histoplasma*, *Blastomyces*, *Paracoccidioides* species are thermally dimorphic fungi causing serious diseases in humans. In addition, many other fungi across the fungal kingdom, including *Talaromyces marneffii*, *Sporothrix schenckii*, *Cryptococcus neoformans*, *C. gattii*, are considered dimorphic and cause significant human diseases. None of these fungi are mentioned in the manuscript. There is a significant amount of transcriptome data published in many of these fungi. If the intent is to look at the evolution of dimorphism or gene families involved in dimorphism, then the integration of some of these datasets should be considered.

-Abstract/background/results: The concept of gene expression is completely misused. Transcriptome studies do not measure gene expression; rather, they measure the abundance of transcripts at a given time in a given condition. The authors provide no evidence to claim some genes are “exclusively yeast expressed,” or “exclusively mycelial expressed,” or “constitutively expressed”. The results can be divided in terms of enrichment of a given transcript in a given state, or changes in abundance; however, RNAseq experiments do not reveal any information on the level of gene expression.

-Fig 1/results: The authors should reconsider the RNAseq analysis especially the determination of the DEGs. There is a significant number of genes with high p-values (closer to bottom) that still pass their significance filter. Perhaps because of that, a very high percentage (~70%) of the genome is labeled as DEG, which is unusually high compared to the numbers in other dimorphic fungi. In addition, pathway analysis revealed no clear enrichment in any pathway, thus it is unclear why Fig1e is included in the main text.

-Fig 2/results: There is an overemphasis on the bidirectional promoters and paralogs showing differential expression in different developmental stages. This is commonly observed in other dimorphic fungi. Not all paralogs are expressed in one form versus the other, and this is perhaps advantageous for the pathogen. The most exciting data in this figure are those in panel b, which show the phenotypes of double mutants, corroborating their phase-specific expression. Surprisingly, these mutants were not used for follow-up RNAseq experiments, but their contribution to the yeast or mycelial transcriptomes would be significant based on their phenotypes.

-Fig 3/results: What are the importance of the motifs identified here? Do these motifs have any predictive power in the existing datasets. For example, does the motif on left found in the majority of the genes with yeast-enriched transcripts?

-Fig 4/results: The figure itself looks aesthetically pleasing; however, there is no significant difference in gene distribution in any groups reported here.

-Fig 5/results: It is unclear why primary mycelium and reverse transition were selected to assay for yeast and hyphae specific transcriptional regulators. A gel image validating the pull-downs would have been good to include here. It is peculiar that the two proteins identified through these assays were not transcription factors. Nevertheless, *df1* seems to contribute to yeast-like growth. However, based on the panel g, its contribution to the transcriptome profiles is not extensive. It is also unclear what the comparison is in panel g (reverse transition versus ?)

-Fig 6/results: This is a nice attempt to include other fungi for comparative analysis; however, the division of orthogroups is not well defined.

(Remarks on code availability)

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In the revised manuscript by Tahiri et al., the authors carefully responded to many of the concerns I have raised earlier and made significant improvements to the manuscript. The addition of the comparative analysis across other dimorphic fungi strengthens the manuscript. With this revision, the scope of the manuscript is broadened.

Minor:

-Line 404-433. Please check all genes, species names for proper italicization and abbreviation.

(Remarks on code availability)

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REVIEWER COMMENTS

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Response: We thank the reviewer for this helpful observation. It is true that, as originally written, the title could lead to confusion. To ensure greater rigor, we have decided to change the title to "Dimorphic Mucorales" instead of "Dimorphic organisms." In addition, we have modified the abstract by explicitly stating that the study focuses on the dimorphism of Mucorales in the second line (line 23). We believe these changes clarify the scope of the article for the reader and improve its accuracy. Moreover, following the suggestions of the other reviewers, we have accurately delimited the work done in Mucorales with respect to the work done in other fungi (see below).

Reviewer #2 (Remarks to the Author):

In the manuscript by Tahiri et al., the authors investigated the molecular basis of dimorphism in the human pathogenic fungus *Mucor lusitanicus*, as well as the conservation of gene families and expression patterns in *Mucor* and *Rhizopus* species within the Mucorales order. Dimorphism in fungi, especially in human pathogenic fungi, is crucial for the transition from an environmental form to a parasitic (host) form. Thus, this study addresses an important question in the fungal field. However, the manuscript offers a moderate conceptual advancement in the field of fungal dimorphism. The experimental approach includes transcriptomics performed with *M. lusitanicus* grown to different

developmental stages (yeast-like, primary mycelium, reverse transition, mature mycelium), gene deletions, and characterization of their effect on transcriptome changes, and pull-down assays using DNA probes. Main concerns are the frequent overinterpretation of results, and the lack of experimental details. The transcriptome results and the generated mutants can be useful to the Mucorales researchers, but the scope remains limited.

We are sincerely grateful to the reviewer for the careful and constructive evaluation of our manuscript. The thoughtful suggestions have greatly helped us improve the clarity, rigor, and, most importantly, the scope of our study.

In the revised version, we have carefully addressed all comments by doing new experiments and analyses, clarifying methodological details, refining figure explanations, and revising terminology for greater precision. Most significantly, following the reviewer's recommendation, we expanded our analyses to include comparisons with a wide range of dimorphic fungi beyond Mucorales. This extension has substantially broadened the scope of the work, transforming it from a study primarily centered on *M. lusitanicus* into one that provides a broader evolutionary perspective on fungal dimorphism.

By incorporating transcriptomic datasets from other medically relevant dimorphic fungi and performing a comprehensive orthogroup analysis across multiple lineages, we now place our findings within a comparative and evolutionary framework. This broader scope makes the results not only more robust but also highly relevant to researchers working on diverse dimorphic fungi and on evolutionary strategies underlying fungal adaptation.

We believe that the revised manuscript now presents a much more comprehensive contribution, with an expanded scope that will be of value to a wider scientific community. We are deeply thankful to the reviewer, whose comments guided us in this direction and allowed us to substantially strengthen the manuscript. Below, a point-by-point response can be found.

Reviewer's comment 1: -Abstract/background: The concept of dimorphism has been overemphasized without the description of many dimorphic fungi. The many members of the Onygenales order, including Coccidioides, Histoplasma, Blastomyces, Paracoccidioides species are thermally dimorphic fungi causing serious diseases in humans. In addition, many other fungi across the fungal kingdom, including *Talaromyces marneffii*, *Sporothrix schenckii*, *Cryptococcus neoformans*, *C. gattii*, are considered dimorphic and cause significant human diseases. None of these fungi are mentioned in the manuscript. There is a significant amount of transcriptome data published in many of these fungi. If the intent is to look at the evolution of dimorphism or gene families involved in dimorphism, then the integration of some of these datasets should be considered.

Response:

We sincerely thank Reviewer 2 for this valuable and constructive comment. This point highlighted a clear shortcoming in the original manuscript, which we have now thoroughly addressed in the revised version. Following the reviewer's suggestion allowed us not only to expand the scope of our analyses but also to make our findings more broadly relevant to researchers working on the wide diversity of dimorphic fungi. Moreover, inspired by this suggestion, we went beyond the original request and carried out an extensive orthogroup

analysis across multiple dimorphic fungi, which further enriched the manuscript. We feel that the resulting changes represent a major step forward for this work, and we are sincerely thankful to the reviewer for guiding us in this direction. These revisions led us to substantially update the *Introduction*, *Results*, *Materials and Methods*, and *Discussion* sections, as well as to include an additional figure (Extended Data Figure 10). Below, we provide a detailed summary of the changes:

Introduction:

We rewrote a subsection of the *Introduction* to emphasize the diversity of dimorphic fungi and their importance in human disease, adding three new references (lines 56–60)^{1–3}.

Results:

As recommended by the reviewer, we incorporated analyses of previously published datasets to examine the main dimorphism-associated gene families identified in our work within other dimorphic fungi. In particular, we analyzed the transcriptomic dataset from *Cryptococcus neoformans* published by Hu et al⁴, focusing on the log2FC values of four gene families used as markers of dimorphism in our study: ferroxidases (*fet3a*, *fet3b*, *fet3c*), permeases (*ftr1a*, *ftr1b*), dismutases (*misda*, *misdb*), and reductases (*nura*, *nurb*) (Extended Data Figure 10a-b). Our results indicate that the dismutase and reductase families in *M. lusitanicus* are not conserved in *C. neoformans*, with only a single ortholog found for each. In contrast, *C. neoformans* harbors expanded ferroxidase and reductase gene families (three and two orthologs, respectively), although only the ferroxidases exhibited differential transcription during dimorphism. These findings reveal both conservation and divergence between *M. lusitanicus* and *C. neoformans*. These results highlight both interesting divergences and points of conservation between *M. lusitanicus* and *C. neoformans*.

Motivated by the reviewer's suggestion to strengthen the manuscript with comparative data, and eager to extend our study even further, we additionally performed a comprehensive orthogroup analysis across 11 thermally dimorphic fungi, including key human pathogens (*Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *P. lutzii*, *Sporothrix schenckii*, *Talaromyces marneffe*, *Cryptococcus neoformans*, *C. gattii*, *Coccidioides posadasii*, *C. immitis*, and *Blastomyces dermatitidis*) (Extended Data Figure 10c–d). Using OrthoFinder, we identified orthogroups across these species and then focused on those containing the dimorphism-related gene families that we had characterized in *M. lusitanicus*. This analysis revealed striking patterns of conservation, expansion, and absence across fungal lineages. Importantly, expansions unique to *M. lusitanicus* and losses in Onygenales species became evident. To visualize these findings, we generated a heatmap summarizing the presence and copy number of these orthogroups across all species (Extended Data Figure 10d).

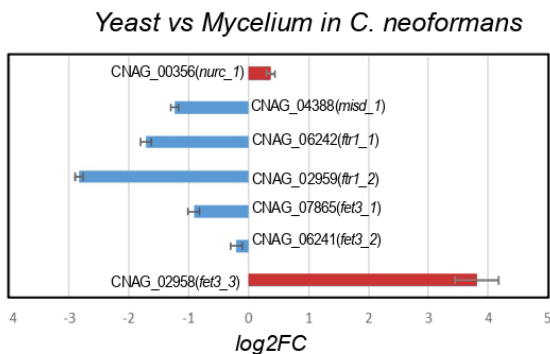
In addition to this global overview, we specifically examined the four marker gene families (*fet*, *ftr*, *nur*, and *misd*) identified in *M. lusitanicus* (Extended Data Figure 10c):

- ***fet* family (OG0000056):** Highly conserved and present in multiple copies in most species, but absent in *H. capsulatum*, *P. brasiliensis*, and *P. lutzii*.
- ***ftr* family (OG0005754):** More restricted, found in *Cryptococcus* spp. and *Mucor*, but absent from other Onygenales members.

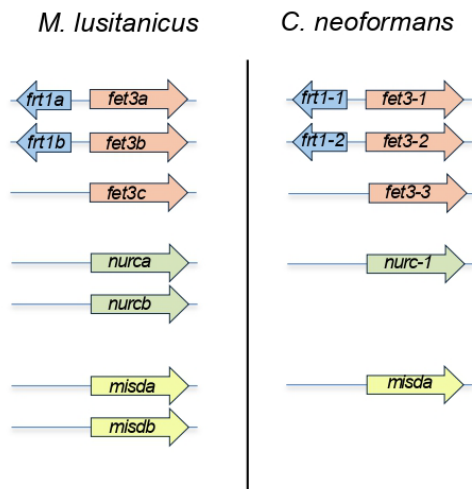
- **nur family (OG0011887):** Appears to be unique to *M. lusitanicus*, suggesting a possible lineage-specific innovation.
- **misd family (OG0001018):** Conserved only in *T. marneffeii*.

Extended Data Figure 10

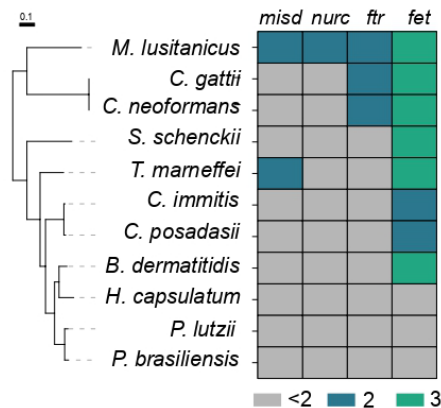
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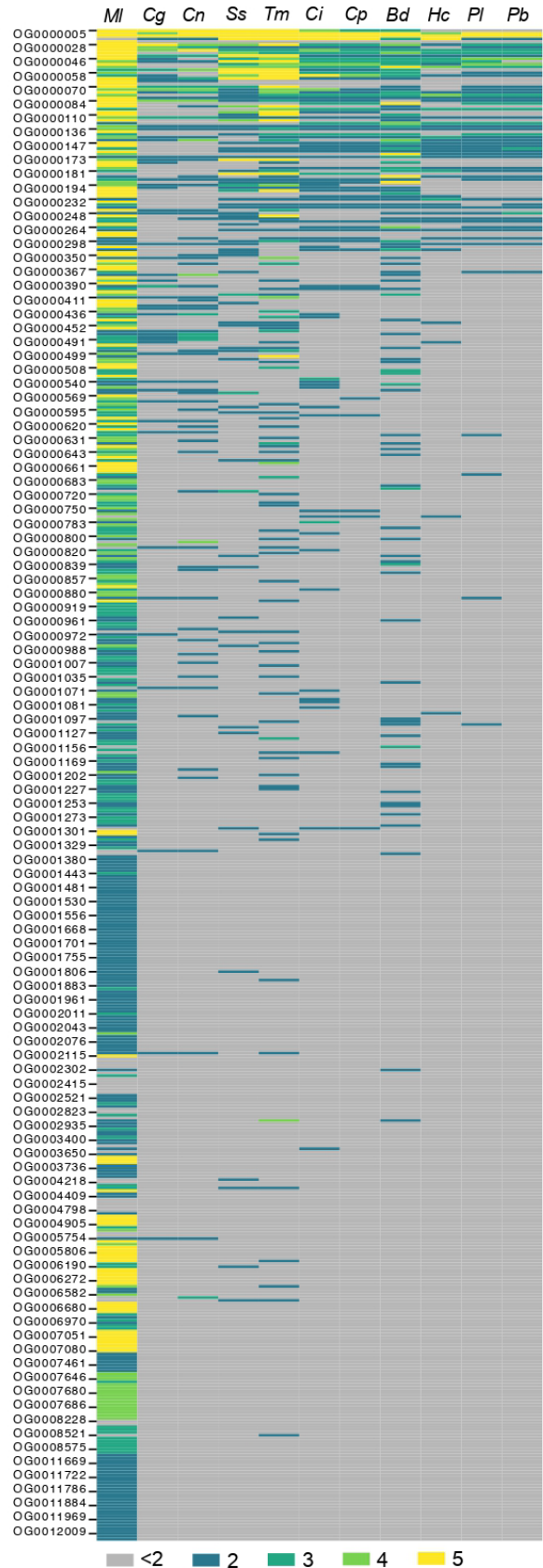
b



c



d



A new section describing these results has been included in the manuscript (lines 404-433)

Discussion:

Our results indicate that most of the dimorphism-associated gene families identified in Mucorales are not conserved in other fungal lineages, suggesting that gene duplication and loss events have played a central role in shaping the diversification of gene families potentially involved in fungal dimorphism. Nevertheless, we identified a reduced core set of orthogroups conserved across multiple groups of dimorphic fungi, including the ferroxidase gene family, for which we confirmed differential expression in *C. neoformans*. These findings suggest that, while the majority of dimorphism-associated gene families in Mucorales are lineage-specific, other dimorphic fungi may rely on distinct sets of specialized gene families that fulfill functions required for their particular modes of dimorphism. Overall, these global patterns underscore both the conservation of a limited set of core orthogroups and the lineage-specific evolutionary trajectories that have shaped the molecular basis of dimorphism in fungi. This new piece of discussion has been included in the manuscript (lines 538-548)

Materials and Methods:

A new subsection entitled “Comparative Analysis of Dimorphism-Related Orthogroups Across Dimorphic Fungi” has been added to the Materials and Methods section (lines 1152-1171)

Reviewer’s comment 2: -Abstract/background/results: The concept of gene expression is completely misused. Transcriptome studies do not measure gene expression; rather, they measure the abundance of transcripts at a given time in a given condition. The authors provide no evidence to claim some genes are “exclusively yeast expressed,” or “exclusively mycelial expressed,” or “constitutively expressed”. The results can be divided in terms of enrichment of a given transcript in a given state, or changes in abundance; however, RNAseq experiments do not reveal any information on the level of gene expression.

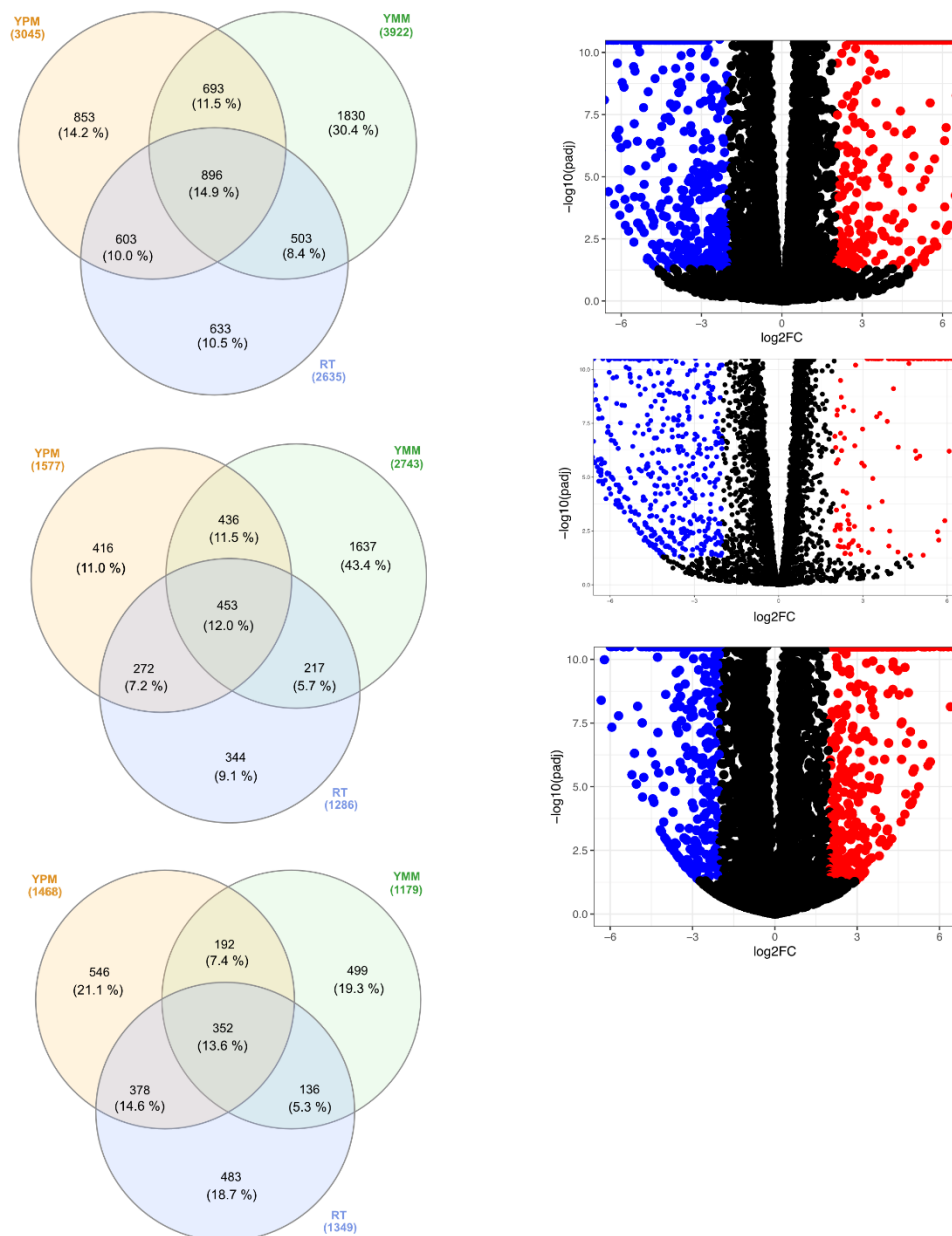
Response: We thank the reviewer for this insightful comment and fully agree with the clarification. Indeed, RNA-seq experiments measure transcript abundance at a specific time and under defined conditions, rather than direct gene expression per se. In the original manuscript, we adopted terminology that is commonly used in RNA-seq studies (e.g., “differentially expressed genes DEGs” or “higher expressed in one condition versus another”), but we recognize that this may cause ambiguity. To address this concern, we have now included an explicit statement early in the text (lines 135–138) clarifying that our analyses are based on transcript abundance across growth conditions. Furthermore, in the Results section (lines 149–153), we revised the wording to specify that “yeast-” or “mycelium-specific” genes refer to those with comparatively higher transcript abundance in the respective conditions. Moreover, we have also included clarifications in the Methods section (lines 994-995). We believe these revisions will improve precision and prevent misinterpretation by readers.

Reviewer’s comment 3: -Fig 1/results: The authors should reconsider the RNAseq analysis especially the determination of the DEGs. There is a significant number of genes with high

pvalues (closer to bottom) that still pass their significance filter. Perhaps because of that, a very high percentage (~70%) of the genome is labeled as DEG, which is unusually high compared to the numbers in other dimorphic fungi. In addition, pathway analysis revealed no clear enrichment in any pathway, thus it is unclear why Fig1e is included in the main text.

Response: We agree with the reviewer that there will be readers interested only in the list of genes that are highly differentially transcribed. Our criteria for defining differentially expressed genes (DEGs) throughout the course of the study took into account those potential readers, but also others who may look for a complete overview of all the genes that are differentially regulated during dimorphism. To address the needs of both types of readers, we ultimately decided to use an $FDR < 0.05$ and $|\log_2 FC| > 1$, and we also included in our supplementary datasets the final lists of DEGs, rather than only the raw data as in many other publications. These supplementary tables include all relevant information and can be easily filtered by the community to generate gene lists using more or less stringent thresholds, depending on specific research needs. Our main objective was to ensure the greatest possible comparability with previously published datasets of gene expression in the species under similar or identical growth conditions (<https://doi.org/10.1038/s41467-024-50365-2>, <https://doi.org/10.3390/ijms22052282>). In addition, to comply with the reviewer's suggestion, we reanalyzed our transcription datasets and regenerated panels 1c and 1d using the more stringent criterion of $|\log_2 FC| > 2$. The updated results are shown below and also included in Supplementary Table 1. We did not detect any major or biologically relevant changes. Although any threshold choice involves some degree of arbitrariness, we believe this approach allows for a robust analysis of transcriptomic data. Regarding Figure 1e, we apologize for the lack of clarity in the original version, as we failed to clearly state that all the pathways shown in the figure are significantly enriched ($FDR < 0.05$). To address this, we have updated the figure legend (line 670), corrected the reference in the text that mistakenly referred to panel 1f (which does not exist) when it should have referred to panel 1e, and clarified the corresponding results in the manuscript (lines 159–163).

Update in Supplementary Table 1



Reviewer's comment 4: -Fig 2/results: There is an overemphasis on the bidirectional promoters and paralogs showing differential expression in different developmental stages. This is commonly observed in other dimorphic fungi. Not all paralogs are expressed in one form versus the other, and this is perhaps advantageous for the pathogen. The most exciting data in this figure are those in panel b, which show the phenotypes of double mutants, corroborating their phase-specific expression. Surprisingly, these mutants were not used for follow-up RNAseq experiments, but their contribution to the yeast or mycelial transcriptomes would be significant based on their phenotypes.

Response: We understand the reviewer's concern and would like to apologize for not expressing the intention of these experiments with sufficient clarity, which may have been a source of confusion in the original manuscript. The genes analyzed in Figure 2 (ferroxidase

and permease gene families) were chosen only as markers to demonstrate that the differential transcription observed in the RNA-seq experiments also has a corresponding functional counterpart in yeast and mycelium. These genes are involved only in iron acquisition, and their mutation affects growth and other processes that require iron, which indirectly might affect the growth of yeast or mycelium if iron is not correctly acquired. In Figure 2, we show that mutations in a specific paralog affect only the growth form in which this paralog is expressed, thereby demonstrating functional specialization of the paralogs. However, these mutants are not directly affected in the regulation of dimorphism, as they can switch between yeast and mycelium correctly. An RNA-seq follow-up of these mutants would reveal only the genes affected by the lack of iron, which is not the purpose of this work. The identification of genes directly involved in the regulation of dimorphism is presented in the second part of the manuscript, where we describe the identification of *dkl* and *dfl* genes, their mutation and phenotype characterization, and their RNA-seq follow-up. All these clarifications have been added to the Results section (lines 174-183).

Reviewer's comment 5: -Fig 3/results: What are the importance of the motifs identified here? Do these motifs have any predictive power in the existing datasets. For example, does the motif on left found in the majority of the genes with yeast-enriched transcripts?

Response: We appreciate this comment as it gives us the opportunity to explain in more detail the biological significance of the identified motifs. The figure shows that the detection of enriched motifs in the promoters of genes with higher transcript abundance in either the yeast or mycelial condition ($\text{FDR} < 0.05$) indicates that these sequence combinations appear more frequently than expected by chance, based on the background base composition. If no motifs were enriched, the analysis would yield no significant result. Therefore, in response to the reviewer's question, yes, for a motif to be considered enriched, it must occur at a higher frequency across all studied promoters in the yeast condition. This suggests that regulatory elements controlling these genes may recognize those sequences. As we understand, this may not have been clearly explained in the original version, we have expanded the figure legend (lines 766–767) as well as the corresponding section in the Results (lines 244–250).

Reviewer's comment 6: -Fig 4/results: The figure itself looks aesthetically pleasing; however, there is no significant difference in gene distribution in any groups reported here.

Response: We thank the reviewer for this valuable observation regarding Figure 4. As correctly noted, this analysis did not reveal striking differences in gene distribution. In response, we have moved the figure to the supplementary material, where it now appears as Extended Data Figure 3. Our intention with this analysis was to explore whether specific genomic regions might be enriched for genes associated with one of the two morphologies, similar to the role of sex chromosomes in vertebrates, or whether, alternatively, these genes are more evenly distributed across the genome. We fully agree with the reviewer that the identification of specialized chromosomes or regions containing dimorphism-related elements would have been more impactful. Nevertheless, we consider the finding that yeast- and mycelium-specific genes are uniformly distributed to be informative, as it supports previous studies suggesting an ancient whole-genome duplication and a broad genomic basis for dimorphism⁵.

Reviewer's comment 7: Fig 5/results: It is unclear why primary mycelium and reverse transition were selected to assay for yeast and hyphae specific transcriptional regulators. A gel image validating the pull-downs would have been good to include here. It is peculiar that the two proteins identified through these assays were not transcription factors. Nevertheless, *dfl* seems to contribute to yeast-like growth. However, based on the panel g, its contribution to the transcriptome profiles is not extensive. It is also unclear what the comparison is in panel g (reverse transition versus ?)

Response: We thank the reviewer for these insightful comments, which give us the opportunity to clarify our results. Primary mycelium and the reverse transition were selected as starting material to represent aerobic and anaerobic conditions, respectively. Our reasoning was that potential mutants in a regulator required to inhibit yeast-cell production under anaerobic conditions could pose challenges for subsequent experiments in these conditions. As it turned out, the regulators we identified, *dfl* and *dql*, indeed affect yeast-cell formation. Importantly, however, the reverse transition can still be induced under anaerobic conditions, which allowed us to further examine the effects of mutations in these genes.

As the reviewer correctly notes, the identified proteins are not transcription factors and do not contain canonical DNA-binding domains. Therefore, they are unlikely to interact directly with promoter DNA, and for this reason, we considered a DNA pull-down validation gel not to be appropriate. Instead, our ongoing work focuses on testing whether these proteins form part of a larger complex and identifying additional components that may mediate DNA interaction, as discussed in the revised manuscript. Moreover, in our purification experiments, the entire protein extract was analyzed by HPLC followed by mass spectrometry, enabling detection of both high- and low-abundance proteins. In contrast, a gel-based approach would primarily visualize only the most abundant proteins, limiting the scope of identification.

Regarding Figure 5g, we appreciate the opportunity to clarify. Our aim was to first illustrate (in the WT column) the set of genes that are differentially expressed during the dimorphic transition, and then to examine how the expression of these same genes changes in the two mutant strains (columns 2 and 3). This design allowed us to highlight how genes differentially regulated in the wild type become deregulated in the mutants. We acknowledge that this was not sufficiently clear in the original text and have revised the Results section accordingly (lines 310–321) to provide a more precise explanation of this analysis.

Reviewer's comment 8: -Fig 6/results: This is a nice attempt to include other fungi for comparative analysis; however, the division of orthogroups is not well defined.

Response: We thank the reviewer for this valuable comment and for highlighting the need for clearer definitions. We realize that we did not provide sufficient explanation of how the orthogroups were defined when additional species were included in the comparative

analysis. As was originally described for the comparison between *M. lusitanicus* and *R. microsporus*, the groups (MG, ME, ML, and NNO) were defined using the same criteria in all subsequent pairwise comparisons (e.g., *M. lusitanicus* vs. *T. elegans*, *M. lusitanicus* vs. *C. recurvatus*). To address this, we have now revised the Results section to explicitly clarify the definition of these groups in the extended analyses (lines 392–394).

References

1. Zhao, Y., Lin, J., Fan, Y. & Lin, X. Life cycle of *Cryptococcus neoformans*. *Annu Rev Microbiol* 73, 17–42 (2019).
2. Wang, F., Han, R. H. & Chen, S. An Overlooked and Underrated Endemic Mycosis—Talaromycosis and the Pathogenic Fungus *Talaromyces marneffe*. *Clin Microbiol Rev* 36, (2023).
3. Van Dyke, M. C. C., Teixeira, M. M. & Barker, B. M. Fantastic yeasts and where to find them: the hidden diversity of dimorphic fungal pathogens. *Curr Opin Microbiol* 52, 55–63 (2019).
4. Hu, P. *et al.* A unique cell wall synthetic response evoked by glucosamine determines pathogenicity-associated fungal cellular differentiation. *PLoS Genet* 17, e1009817 (2021).
5. Corrochano, L. M. *et al.* Expansion of Signal Transduction Pathways in Fungi by Extensive Genome Duplication. *Current Biology* 26, 1577–1584 (2016).

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