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Decoding the chemical language of Suillus fungi: genome mining and untargeted metabolomics uncover terpene chemical diversity

Sameer Mudbhari, Lotus Lofgren, Manasa Appidi, Rytas Vilgalys, Robert Hettich, and Paul Abraham

Corresponding Author(s): Paul Abraham, Oak Ridge National Laboratory

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1128/msystems.01225-23

Re: mSystems01225-23 (Decoding the chemical language of Suillus fungi: genome mining and untargeted metabolomics uncover terpene chemical diversity)

Dear Dr. Paul E. Abraham:

Thank you for the privilege of reviewing your work. Below you will find my comments, instructions from the mSystems editorial office, and the reviewer comments.

Please return the manuscript within 60 days; if you cannot complete the modification within this time period, please contact me. If you do not wish to modify the manuscript and prefer to submit it to another journal, notify me immediately so that the manuscript may be formally withdrawn from consideration by mSystems.

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• Upload a compare copy of the manuscript (without figures) as a "Marked-Up Manuscript" file
• Upload a clean .DOC/.DOCX version of the revised manuscript and remove the previous version
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Thank you for submitting your paper to mSystems.

Sincerely,
Yu-Liang Yang
Editor
mSystems

Reviewer #1 (Comments for the Author):

Please find the attachment.

Reviewer #2 (Comments for the Author):

In this study, Mudbhari et al conduct an in-depth investigation into the exometabolomes of three Suillus species, both in pure culture and co-culture. As expected, they find that many new metabolites are produced when different isolates are grown in co-culture with each other. The authors do a great job summarizing a lot of metabolomic data, and I commend them on a clear and

succinct manuscript. I have just a few comments and suggestions that I hope would improve the manuscript.

180-203: I know similar antiSMASH analyses have previously been published, but since new analyses are being reported here, please provide all data associated with the BGC predictions in these genomes as supplementary info (e.g., GFF files of all predicted clusters and their genomic coordinates, antiSMASH HTML files, all bigSCAPE files showing GCF assignments for each BGC etc etc).

Figure 5: Including all "no matches" nodes makes it difficult to appreciate and visualize the network structure. I suggest moving panel A to the supplement doing one of two things for the main figure to simplify the network for visualization purposes: either remove all networks containing fewer than 10 nodes (or some other reasonable threshold) or remove all networks consisting entirely of nodes with "no matches".

Given all the incredible data presented here, I think a couple of additional statistical analyses that might increase interest in this study are justified:

Figure 4A: the heatmap makes it difficult to appreciate whether changes in relative abundance are due to additive or interactive effects between co-cultured fungi but this would seem important to determine given the objectives of this study. e.g., does methylpentanoic acid have a higher abundance in VC-EM16 co-culture simply because its production in VC and its production in EM16 are being added together or is there more of this compound than you would expect? Please explore statistical tools like a generalized linear model or consult with a statistician about another appropriate method where you can estimate additive and non-additive variance in terpene abundance as a function of species 1, species 2, and species 1 x species 2

Figure 3: Its very useful to see in some of the other figures how metabolites break down according to treatment (e.g., figure 2c). Given the focus on suilllus chemical ecology, I think a similar breakdown would be interesting in this figure where you specifically examine different chemical classes. For example, how many chemicals in each class are only produced in monoculture vs co-culture? As a complement to this bar graph, is there a way to calculate which combinations of fungi produce the greatest diversity of metabolite chemical classes? Is it possible to calculate and compare alpha and beta "chemical" diversity and compare them between treatments? Does this in any way correlate with the number of BGCs in the interacting genomes?

Mudbhari et al. performed untargeted metabolomics of three *Suillus* species, an important genus of ectomycorrhizal fungi. The study describes the mass spectrometric analysis of metabolites produced by the three species grown individually and in co-culture. The authors attempted to identify and/or classify the metabolites with two different approaches that are both well-established in the field.

The structure and language of this manuscript is clear and easy to follow. I appreciate that the authors are very cautious in interpreting their results, since they did not identify the metabolites by NMR. However, this makes the study seem rather incomplete and shallow. The authors did not even speculate on the biosynthetic origin of the metabolites although they point out that there are multiple biosynthetic gene clusters in the genomes of these species. The BGC analysis, is a repetition of a previous analysis when the genomes were first published.

In addition to this lack of novelty/innovation, my major concern is that the methods section is very brief and does not properly explain how the samples were prepared (replicates, controls, etc.) and how the analysis of the metabolomics data was performed (blanks, QC, etc.).

Minor concerns:
Line 117: what exactly was the input for BiG-SCAPE?

Line 122: where did the specimen come from? Are the strains identical with the ones sequenced in ref 12?

Line 125: Was each experiment performed with 5 petri dishes and then analyzed in 3 replicates, so 15 replicates in total? I find it confusing that the 5 replicates are called technical, since I would consider those biological and the other 3 technical replicates.

Line 169: I would expect a separate methods section explaining the data processing (QC, background subtraction, etc.) and analysis, as well as the statistical analysis.

Line 193: Why are the numbers of NRPS-like and terpene BGCs in panel C and D different from panel B?

Line 211: Unclear description of the steps of data analysis and the rationale behind it. The procedure should be properly explained in the methods section. What was the rationale for analyzing the data with ChemSpider and then excluding it afterwards?

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Reply to reviewer's comments:

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Response to reviewer: We thank the reviewer for their critical evaluation of our work and the thoughtful remarks and constructive criticisms. We understand that there are additional measurements and experiments that can be performed to define the structure of the compounds and their relatedness to the predicted biosynthetic gene clusters. We agree with the reviewer that those pursuits are warranted. Although we do intend to further characterize many of the resulting compounds observed, those experiments (e.g., NMR analysis and genome engineering) do require a fair amount of additional time, resources, and energy that fall outside the scope of this study. At this point in time, we prefer to not speculate as to what metabolites match against any particular antiSMASH prediction. This is largely because predicted backbone genes, such as terpene synthase, could lead to the production of a variety of terpene molecules.

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Response to reviewer: While we did perform BGC analysis in our previously published research article (Lofgren et al., New Phytologist, 2021) and in this study, the analysis and outcome are distinct. In the already published study, our comparative genomics analysis investigated BGC predictions and their similarities across many genera of ECM fungi. In this study, we instead investigated BGC predictions and their similarities across three distinct *Suillus* species. Here, this study revealed that there is inter-species variability in the encoded biosynthetic gene clusters associated with secondary metabolite production. To further clarify this point, we have revised the manuscript text on page 6, line 231-254.

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Response to reviewer: We subset the data for PCA to only focus on those metabolites have tandem MS data and a putative identification to our spectral libraries. This decision to only focus on putative metabolite identifications was to be consistent and more accurate with our other downstream analyses. We understand that this rationale was not clear because we did not properly explain how we were addressing “redundancies” in the data. To address this, we have added additional text to explain our rationale for why the number of putative metabolite identifications was reduced from 3,769, and this can be found on page 7, line 267. Additionally, we addressed confusion related to the usage of “biological” and “technical” replicates in a previous comment.

Response to reviewer:

Reviewer 1 Line 274: Do all reference compounds also elute with the same retention time? Line 303: typo “4D”?

Response to reviewer: That is a great question and could help with classification. Unfortunately, compounds had similar elution times.

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Response to reviewer: We corrected this typo on page 9, line 356.

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Response to reviewer: As mentioned in a previous comment to the reviewer, we used five replicates of each culture conditions. We have revised the manuscript to make it clear to understand and these changes can be found on page 125-127.

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Response to reviewer: As suggested, we have revised the text page 12, line 482.

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Response to reviewer: For each compound, the average intensity across biological replicates was Log10-transformed into a scaled value (standard z-score). This data transformation was performed to accommodate the large range of values measured. We have revised the manuscript by adding this information to the figure legend on page 12, line 280-281.

Reviewer #2 (Comments for the Author):

Reviewer 2 In this study, Mudbhari et al conduct an in-depth investigation into the exometabolomes of three Suillus species, both in pure culture and co-culture. As expected, they find that many new metabolites are produced when different isolates are grown in co-culture with each other. The authors do a great job summarizing a lot of metabolomic data, and I commend them on a clear and succinct manuscript. I have just a few comments and suggestions that I hope would improve the manuscript.

Response to reviewer: We thank the reviewer for their thoughtful remarks and constructive criticism.

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Response to reviewer: We have provided the requested information as new supplementary data files detailed in the 'Data Availability' statement. Further, this information has been provided in revised manuscript on page 5, line 249-250.

Reviewer 2 Figure 5: Including all "no matches" nodes makes it difficult to appreciate and visualize the network structure. I suggest moving panel A to the supplement doing one of two things for the main figure to simplify the network for visualization purposes: either remove all networks containing fewer than 10 nodes (or some other reasonable threshold) or remove all networks consisting entirely of nodes with "no matches".

Response to reviewer: We thank the reviewer for the feedback on how to best present these results. We have revised Figure 5, as suggested, by moving this version to Supplemental Material (Figure S4). We have replaced the original with a revised version that filtered out all subnetworks containing fewer than 6 nodes. We decided to keep the nodes with "no matches" because we view these as an important feature of the data generated.

Reviewer 2 Figure 4A: the heatmap makes it difficult to appreciate whether changes in relative abundance are due to additive or interactive effects between co-cultured fungi but this would seem important to determine given the objectives of this study. e.g., does methylpentanoic acid have a higher abundance in VC-EM16 co-culture simply because its production in VC and its production in EM16 are being added together or is there more of this compound than you would expect? Please explore statistical tools like a generalized linear model or consult with a statistician about another appropriate method where you can estimate additive and non-additive variance in terpene abundance as a function of species 1, species 2, and species 1 x species 2

Author's reply: We agree with the reviewer that it would be helpful to dissect the amount of compound signal being provided by each organism present in the coculture. That is, what amount of increased signal in coculture could explained by either a combined signal sourced from both organisms (additive) or by enhanced gene expression of a single organism (interactive). We have carefully considered this comment; however, our expectation is that those determinations (i.e., additive effects) are best made through alternative approaches, such as stable isotope labeling, and additional experimentation.

Reviewer 2 Figure 3: Its very useful to see in some of the other figures how metabolites break down according to treatment (e.g., figure 2c). Given the focus on suilllus chemical ecology, I think a similar breakdown would be interesting in this figure where you specifically examine different chemical classes. For example, how many chemicals in each class are only produced in monoculture vs co-culture? As a complement to this bar graph, is there a way to calculate which combinations of fungi produce the greatest diversity of metabolite chemical classes? Is it possible to calculate and compare alpha and beta "chemical" diversity and compare them between treatments? Does this in any way correlate with the number of BGCs in the interacting genomes?

Author's reply: We thank the reviewer for the thoughtful comments. We have created new figures (Figures S2 and S3) to address these comments for the organic and aqueous fractions. These additional UpSet plots illustrate the overlap in chemical classes for the observed metabolites in each sample.

Re: mSystems01225-23R1 (Decoding the chemical language of Suillus fungi: genome mining and untargeted metabolomics uncover terpene chemical diversity)

Dear Dr. Paul E. Abraham:

Thank you for the privilege of reviewing your work. The manuscript has been revised accordingly and addresses the feedback provided by the reviewers. While you have made significant progress, there are several minor issues that still require attention prior to formal acceptance. Kindly refer to the annotations in the attached document for further details.

Please return the manuscript as soon as possible; if you cannot complete the modification within this time period, please contact me. If you do not wish to modify the manuscript and prefer to submit it to another journal, notify me immediately so that the manuscript may be formally withdrawn from consideration by mSystems.

Revision Guidelines
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Thank you for submitting your paper to mSystems.

Sincerely,
Yu-Liang Yang
Editor
mSystems

Reply to editor:

As requested, we have addressed the highlighted marks in the manuscript.

Reply to reviewer's comments:

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Response to reviewer: For each compound, the average intensity across biological replicates was Log10-transformed into a scaled value (standard z-score). This data transformation was performed to accommodate the large range of values measured. We have revised the manuscript by adding this information to the figure legend on page 12, line 280-281.

Reviewer #2 (Comments for the Author):

Reviewer 2 In this study, Mudbhari et al conduct an in-depth investigation into the exometabolomes of three Suillus species, both in pure culture and co-culture. As expected, they find that many new metabolites are produced when different isolates are grown in co-culture with each other. The authors do a great job summarizing a lot of metabolomic data, and I commend them on a clear and succinct manuscript. I have just a few comments and suggestions that I hope would improve the manuscript.

Response to reviewer: We thank the reviewer for their thoughtful remarks and constructive criticism.

Reviewer 2 180-203: I know similar antiSMASH analyses have previously been published, but since new analyses are being reported here, please provide all data associated with the BGC predictions in these genomes as supplementary info (e.g., GFF files of all predicted clusters and their genomic coordinates, antiSMASH HTML files, all bigSCAPE files showing GCF assignments for each BGC etc etc).

Response to reviewer: We have provided the requested information as new supplementary data files detailed in the 'Data Availability' statement. Further, this information has been provided in revised manuscript on page 5, line 249-250.

Reviewer 2 Figure 5: Including all "no matches" nodes makes it difficult to appreciate and visualize the network structure. I suggest moving panel A to the supplement doing one of two things for the main figure to simplify the network for visualization purposes: either remove all networks containing fewer than 10 nodes (or some other reasonable threshold) or remove all networks consisting entirely of nodes with "no matches".

Response to reviewer: We thank the reviewer for the feedback on how to best present these results. We have revised Figure 5, as suggested, by moving this version to Supplemental Material (Figure S4). We have replaced the original with a revised version that filtered out all subnetworks containing fewer than 6 nodes. We decided to keep the nodes with "no matches" because we view these as an important feature of the data generated.

Reviewer 2 Figure 4A: the heatmap makes it difficult to appreciate whether changes in relative abundance are due to additive or interactive effects between co-cultured fungi but this would seem important to determine given the objectives of this study. e.g., does methylpentanoic acid have a higher abundance in VC-EM16 co-culture simply because its production in VC and its production in EM16 are being added together or is there more of this compound than you would expect? Please explore statistical tools like a generalized linear model or consult with a statistician about another appropriate method where you can estimate additive and non-additive variance in terpene abundance as a function of species 1, species 2, and species 1 x species 2

Author's reply: We agree with the reviewer that it would be helpful to dissect the amount of compound signal being provided by each organism present in the coculture. That is, what amount of increased signal in coculture could explained by either a combined signal sourced from both organisms (additive) or by enhanced gene expression of a single organism (interactive). We have carefully considered this comment; however, our expectation is that those determinations (i.e., additive effects) are best made through alternative approaches, such as stable isotope labeling, and additional experimentation.

Reviewer 2 Figure 3: Its very useful to see in some of the other figures how metabolites break down according to treatment (e.g., figure 2c). Given the focus on suilllus chemical ecology, I think a similar breakdown would be interesting in this figure where you specifically examine different chemical classes. For example, how many chemicals in each class are only produced in monoculture vs co-culture? As a complement to this bar graph, is there a way to calculate which combinations of fungi produce the greatest diversity of metabolite chemical classes? Is it possible to calculate and compare alpha and beta "chemical" diversity and compare them between treatments? Does this in any way correlate with the number of BGCs in the interacting genomes?

Author's reply: We thank the reviewer for the thoughtful comments. We have created new figures (Figures S2 and S3) to address these comments for the organic and aqueous fractions. These additional UpSet plots illustrate the overlap in chemical classes for the observed metabolites in each sample.

Re: mSystems01225-23R2 (Decoding the chemical language of Suillus fungi: genome mining and untargeted metabolomics uncover terpene chemical diversity)

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Your manuscript has been accepted, and I am forwarding it to the ASM production staff for publication. Your paper will first be checked to make sure all elements meet the technical requirements. ASM staff will contact you if anything needs to be revised before copyediting and production can begin. Otherwise, you will be notified when your proofs are ready to be viewed.

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Yu-Liang Yang
Editor
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