The ISME Journal (2021) 15:2173–2179
https://doi.org/10.1038/s41396-021-00924-y

COMMENT

Regulation of mating genes during arbuscular mycorrhizal isolate co-existence—where is the evidence?

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Received: 6 October 2020 / Revised: 23 January 2021 / Accepted: 1 February 2021 / Published online: 2 March 2021

Abstract

A recent study published by Mateus et al. [1] claimed that 18 "mating-related" genes are differentially expressed in the model arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* when genetically distinct fungal strains co-colonize a host plant. To clarify the level of evidence for this interesting conclusion, we first aimed to validate the functional annotation of these 18 *R. irregularis* genes using orthology predictions. These analyses revealed that, although sequence relationship exists, only 2 of the claimed 18 *R. irregularis* mating genes are potential orthologues to validated fungal mating genes. We also investigated the RNA-seq data from Mateus et al. [1] using classical RNA-seq methods and statistics. This analysis found that the over-expression during strain co-existence was not significant at the typical cut-off of the *R. irregularis* strains DAOM197198 and B1 in plants. Overall, we do not find convincing evidence that the genes involved have functions in mating, or that they are reproducibly up or down regulated during co-existence in plants.

Significance of the claim for regulated mating genes in AMF

Arbuscular mycorrhizal fungi (AMF) are keystone mutualists in terrestrial ecosystems, as they improve plant yields and protect their hosts against pathogens [2]. These fungi are also genetic oddballs, as they carry thousands of nuclei in a large syncytium at all times [3]. This constant multinucleate state was proposed to have helped AMF evolve for close to a billion year in the absence of sex [4], but this hypothesis is challenged by the discovery of compelling signatures of sexual reproduction in these organisms. Specifically, all AMF carry meiosis and mating-related genes [5] and genome-based evidence for inter-strain recombination [6]. Furthermore, some cultured strains show a dikaryotic-like nuclear organization where two parental nuclear genotypes co-exist in the mycelium [7, 8]. However, despite this evidence, sexual reproduction, i.e., mating and plasmogamy producing a recombined haploid progeny through meiosis [7, 9] has not been observed in these organisms.

Mateus et al. [1], recently investigated transcriptional responses in AMF fungi during the strain co-existence in plants using RNA-seq and concluded that several genes involved in mating were up or down regulated (see Tables 1 and 2 in Mateus et al. [1]). If true, this finding would represent the first direct evidence for mechanistic processes related to mating in AMF [1].

AMF genomes are very large compared to most fungal relatives [3] and contain highly expanded gene families, including an overrepresentation of genes involved in signaling pathways and protein–protein interactions compared with known fungal gene repertoires [6, 10–12]. The use of sequence homology to attribute specific functions to genes that are members of large and functionally diverse families is problematic, especially in AMF where large genomes include many expanded gene families [12, 13]. The difficulty concerns discriminating between orthologues, which are genes in different species that evolved from a common ancestral gene (i.e., are monophyletic), and paralogs, which result from duplications (i.e., can belong to distinct

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41396-021-00924-y.

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Table 1 Best reciprocal hit for the *R. irregularis* genes claimed to be involved in mating from the MycoCosm database.

Rhizophagus irregularis gene query	# of hits in the JGI fungal database (limit of 10,000)	Fungal mating gene highlighted by Mateus et al. [1]	Is the proposed fungal gene found in JGI database (>98% prot id)	Reciprocal best hits analysis			
				Mycocosm reciprocal best hits	% idenity	*e*-value	Alignment score
GBC10892.1	8	KZV10725.1	Yes	jgi|Fimjon1|161196|CE161195_3861 GTPase IMAP family member 4-like	30.1	3.0e −10	63.2
GBC19598.1	4299	AAD42946.1	Yes	jgi|Thasp1|29113|gm1.2107_g hypothetical protein PTT_16366	46.6	1.9e −40	163.7
GBC21696.1	4399	KZV11616.1	Yes	jgi|Suipla1|1074914| fgenesh1_kg.6_#_1318_#_TRINITY_DN7005_c0_g2_i6 kinase-like protein	33.5	3.9e −17	87.4
GBC21938.1	1739	CAA84882.1	Yes	jgi|MorAD185_1_1|328570| fgenesh1_kg.2_#_3333_#_TRINITY_DN4015_c0_g1_i1 kinase-like protein	31.5	6.9e −21	100.5
GBC21972.1	10000	KZV12764.1	Yes	jgi|Mycgal1|1633127|e_gw1.296.23.1 Amp- ligase	33.9	4.9e −22	102.4
GBC27006.1	15	ABS85543.1	Yes	jgi|Glopo1|640658|MIX13823_94_26 Hypothetical protein with MYND-type motif	26.2	2.9e −38	157.9
GBC27247.1	10000	KZV07375.1	Yes	jgi|Mychae1|1111605| fgenesh1_kg.38_#_306_#_TRINITY_DN7147_c0_g1_i32 Protein kinase	29.2	4.9e −21	101.3
GBC28192.1	3031	KZV11281.1	Yes	jgi|Synplu1|668182| fgenesh1_kg.2_#_1315_#_TRINITY_DN2864_c0_g1_i1 High affinity methionine permease	30.1	1.7e −56	218.4
GBC28793.1	10000	KZV12646.1	Yes	jgi|Umbelo1|418631|e_gw1.2.1617.1 Cytochrome P450	32.6	1.0e −74	278.9
GBC31594.1	94	ADT91565.1	Yes	jgi|Trapub1|4930|scaffold_1563.4_gi|338818278|sp|C7U331.1| MATMC_SCHPM High Mobility Group Protein	35.5	9.0e −08	56.2
GBC31744.1	6480	ADU02296.1	No genome in mycocosm; Genbank RBH: PKY26566.1	jgi|Lobtra1|383738|estExt_fgenesh1_pg.C_40055 Hypothetical Protein with helicase domain	36.8	6.6e −226	783.1
GBC37885.1	33	AET35419.1	No genome in mycocosm; Genbank RBH: XP_025169424.1	jgi|Chyhya1|94423|CE94422_223 High Mobility Group Protein	31.9	8.9e −09	59.7
GBC38036.1	5767	KZV07303.1	Yes	jgi|Catan2|1285371|CE285371_1765 Putative Protein kinase	27.6	2.2e −24	111.7
GBC40214.1	8443	XM751538.1	Accession number absent from NCBI	jgi|Xentul1|1117655|CE1117654_5253 Putative Protein kinase	25.8	7.4e −19	93.6
GBC46658.1	6730	ADU02296.1	No genome in mycocosm; Genbank RBH: PKY26566.1	jgi|Disorn1|444180|estExt_Genemark1.C_3190003 Hypothetical Protein with helicase domain	33.2	0.0e +00	1129.8

Table 1 (continued)

Rhizophagus irregularis gene query	# of hits in the JGI fungal database (limit of 10,000)	Fungal mating gene highlighted by Mateus et al. [1]	Is the proposed fungal gene found in JGI database (>98% prot id)	Reciprocal best hits analysis			
				Mycocosm reciprocal best hits	% idenity	*e*-value	Alignment score
GBC47027.1	4767	KZV07361.1	Yes	jgi|Rhisa1|933973|estExt_Genemark1.C_2510018 Putative Protein kinase	24.9	6.6e−21	99.4
GBC47251.1	2387	KZV10712.1	Yes	jgi|Gilper1|522078| fgenesh1_kg.2_#_78_#_TRINITY_DN5509_c3_g1_i2 Putative Protein kinase	25.6	9.8e−21	99.4
GBC53331.1	482	AFA26123.1	No genome in mycocosm; Genbank RBH: XP_025169424.1	jgi|Daequ1|385277|CE385276_835 High Mobility Group Protein	32.8	7.0e−12	69.7

RBH search targets include all genes listed in Tables 1 and 2 from Mateus et al. [1].

paraphyletic clades). Orthologues generally retain the same function during the course of evolution, while paralogues facilitate functional innovation by removing evolutionary constraints on conserved functions [14, 15]. Therefore, clarification of evolutionary relationships is an essential step for reliable prediction of gene function in silico, and overlooking this step most often leads to spurious gene predictions.

Since orthology analyses were not clearly described by Mateus et al. [1], we first sought to clarify the "homology status" of the genes listed in Tables 1 and 2 from Mateus et al. [1], as these are claimed to be involved in mating and differentially expressed in *R. irregularis* during strain co-existence [1]. To do so, we used two approaches that provide gene orthology prediction between species [16] (Supplemental Methods). The best candidate orthologues we identified differ from those identified by Mateus et al. [1]. We then re-analyzed the RNA-seq data for evidence of differential expression of both the originally claimed "mating gene homologs" and the "best match orthologs" of validated mating genes from our analysis using stringent statistical thresholds. We conclude that there is no significant support for mating in this data set.

Best reciprocal hits and OrthoMCL analysis of *R. irregularis* genes claimed to be involved in mating

Attribution of functions to differentially expressed genes in the *R. irregularis* genome by Mateus et al. was based on their similarity to known fungal mating genes show surprisingly low statistical significance. For example, the *R. irregularis* gene GBC47251.1, which is claimed by Mateus et al. to represent the key mating gene STE20, shows an *e*-value of only 5e−12 and an amino-acid sequence identity of only 26.57% against the *Saccharomyces cerevisiae* STE20 gene (KZV10712.1) used in their comparison. However, this attribution is not justified since GBC47251.1 is not the closest match to STE20. When STE20, a validated fungal mating gene, is used as the query sequence this shows that other *R. irregularis* genes are significantly more similar—e.g., the *R. irregularis* accession GBC37837.1 has *e*-value 7e−139 and 41% identity. Given this, we were concerned many of the putative genes may represent distant paralogues of fungal mating genes and systematically assessed the support for functional attribution from sequence relationship.

To assess the potential for paralogy to confound the interpretation of mating functions we used the 18 putative mating genes identified by Mateus et al., as query sequences against the high-quality protein databases from the JGI Mycocosm Rhiir2 [17]. The best hits emerging from our

Table 2 Alternative best hit genes from GBC data set used by Mateus et al. [1] using reciprocal blast.

Fungal mating gene highlighted by Mateus et al. [1]	*R. irregularis* fungal mating gene proposed by Mateus et al. to be related to fungal mating genes in column 1	Best hit against genome database in MycoCosm	Species	Actual best hit of column #1 against the database used by Mateus et al. [1]
AAD42946.1	GBC19598.1	Aspnid1_GeneCatalog_proteins_20110130.aa.fasta	*Aspergillus nidulans*	GBC26474.1
ABS85543.1	GBC27006.1	Ustma2_2_GeneCatalog_proteins_20171117.aa.fasta	*Ustilago maydis*	GBC19644.1
ADT91565.1	GBC31594.1	Rhior3_proteins.fasta	*Rhizopus delemar*	GBC35980.1
ADU02296.1	GBC31744.1	The genome is not in MycoCosm	*Rhizopus oryzae*	–
ADU02296.1	GBC46658.1	The genome is not in MycoCosm	*Rhizopus oryzae*	–
AET35419.1	GBC37885.1	The genome is not in MycoCosm	*Syzygites megalocarpus*	–
AFA26123.1	GBC53331.1	The genome is not in MycoCosm	*Mucor mucedo*	–
CAA84882.1	GBC21938.1	SacceYB210_1_GeneCatalog_proteins_20130329.aa.fasta	*Saccharomyces cerevisiae*	GBC19644.1
KZV07303.1	GBC38036.1	SacceYB210_1_GeneCatalog_proteins_20130329.aa.fasta	*Saccharomyces cerevisiae*	GBC39969.1
KZV07361.1	GBC47027.1	SacceYB210_1_GeneCatalog_proteins_20130329.aa.fasta	*Saccharomyces cerevisiae*	GBC36301.1
KZV07375.1	GBC27247.1	SacceYB210_1_GeneCatalog_proteins_20130329.aa.fasta	*Saccharomyces cerevisiae*	GBC41709.1
KZV10712.1	GBC47251.1	SacceYB210_1_GeneCatalog_proteins_20130329.aa.fasta	*Saccharomyces cerevisiae*	GBC26253.1
KZV10725.1	GBC10892.1	SacceYB210_1_GeneCatalog_proteins_20130329.aa.fasta	*Saccharomyces cerevisiae*	GBC48907.1
KZV11281.1	GBC28192.1	SacceYB210_1_GeneCatalog_proteins_20130329.aa.fasta	*Saccharomyces cerevisiae*	NO HIT
KZV11616.1	GBC21696.1	SacceYB210_1_GeneCatalog_proteins_20130329.aa.fasta	*Saccharomyces cerevisiae*	GBC41557.1
KZV12646.1	GBC28793.1	SacceYB210_1_GeneCatalog_proteins_20130329.aa.fasta	*Saccharomyces cerevisiae*	GBC29961.1
KZV12764.1	GBC21972.1	SacceYB210_1_GeneCatalog_proteins_20130329.aa.fasta	*Saccharomyces cerevisiae*	GBC48685.1

analysis showed that each of the claimed mating genes is a member of a large fungal gene family of broad function—i.e., protein kinases, cytochrome oxidases, etc., as can be seen from the number of hits in the JGI fungal database recorded in column 2 of Table 1. A similar analysis was then conducted using fungal mating genes that were reported by Mateus et al. as being homologous to the upregulated *R. irregularis* genes (see Table 2, column 3, in Mateus et al. [1]). In this analysis none of the supposed mating genes was the best hit to the reference fungal mating gene—e.g., using AAD42946.1 as query should find GBC19598.1 as first hit, instead in our analyses it retrieved GBC26474.1 (Table 2, column 5). To challenge these findings with a different approach, we used OrthoMCL [18] (Supplemental Methods) to identify functional clusters of orthologs (and recent paralogs) that include both validated fungal mating genes and *R. irregularis* genes contained in either the GBC database used by Mateus et al. [1], or the JGI Mycocosm Rhiir2 database [17].

The OrthoMCL analysis revealed that only 7 of the 18 genes listed by Mateus et al. [1] share such clusters (Supplemental Table 1). Based on OrthoMCL only 2 of these 7 cases are the putative *R. irregularis* orthologue candidate of fungal mating genes (e.g., GBC28192.1, and GBC28793.1; Supplemental Table 1).

In summary, our analysis suggests two significant weaknesses. The best matches to validated mating genes have not been identified in *R. irregularis* (did not find candidate orthologs) and of the genes identified by differential expression the predictions of function are not based on demonstrated orthology and are therefore not supported by available evidence.

Regulation of proposed mating-related AMF genes using conventional RNA-seq analyses and statistical thresholds

The above mentioned findings do not exclude the possibility that the genes identified by Mateus et al. [1] are involved in mating, rather they clarify that their supporting arguments are based on spurious evolutionary relationships. To explore further the evidence for differential expression under the specific conditions of co-existence, we also aimed to validate their differential expression when the *R. irregularis* strains DAOM197198 and B1 colonize the same plant host.

Mateus et al. [1] claimed that 18 genes are differentially expressed during strain co-existence. We note, however, that some of the same genes have a different expression response across strains under the same condition—i.e., upregulated in the strain DAOM197198 but downregulated in B1 (e.g., GBC53331.1, GBC31594.1, GBC37885.1) in their study with *p*-value cut-off 0.1. In our view, the claim that mating genes are differentially expressed during strain co-existence should show consistency in expression in both strains (biological replication). Specifically, if mating processes are really underway in planta, then the same genes should be either consistently upregulated or downregulated across similar conditions—i.e., genes should not be subjected to random regulation as suggested, for example, by data available in Supplemental Table 6 from Mateus et al. [1]. Furthermore, we note that Mateus et al. [1], using an adjusted value of <0.1 as a threshold to claim that transcript changes are significant. Given that transcript level validation was not performed by Mateus et al. [1], for example, using digital droplet or qPCR, for at least a subset of the 18 genes they highlight, we believe that an adjusted value of <0.1 could result in a substantial number of false positives; particularly given the large number genes analyzed in the *R. irregularis* genome. As such, given the importance of the claims reported by Mateus et al. [1], a more canonical adjusted value of <0.05 is needed to conclusively support evidence of gene regulation using their RNA-seq data set.

To test the support for expression changes in the 18 putative mating genes during co-existence, we re-analyzed their RNA-seq data using Deseq2 with the following basic assumptions: to be deemed regulated a gene should be (i) differentially expressed in both co-inoculation treatments compared to DAOM197198 or B1 alone; (ii) differentially expressed identically in both conditions and (iii) show regulation at the adjusted value of <0.05. Using this approach revealed that only two *R. irregularis* genes (GBC31744.1, GBC38036.1) out of 18 proposed by Mateus et al. [1] to be involved in mating show evidence of differential expression in both comparisons—i.e., in both co-inoculation treatments compared to DAOM197198 or B1 alone (Supplemental Table 2). However, neither of these two genes share clades with validated fungal mating genes and thus, in our opinion, should not be considered mating genes.

Our analysis had identified other candidate *R. irregularis* mating genes using OrthoMCL (Supplemental Table 1), so we also tested if these were differentially expressed in both co-inoculation treatments. This analysis revealed changes for two putative orthologues of mating genes (Rhiir2_1|1633285, Rhiir2_1|1616235, Supplemental Tables 1 and 2), however, one was upregulated and one downregulated. During mating, recombination occurs and triggers the expression of meiosis genes [5, 19]. As such, AMF strain co-existence should lead to the upregulation of known meiosis-specific AMF genes (MSG) if mating is present. We tested this hypothesis by investigating gene expression of *R. irregularis* meiosis-specific genes (MSG) using the above-mentioned approach. We found that MSG are expressed at very low levels—i.e., most have no mapped read or are not

significantly and conservatively expressed across conditions during strain co-existence (Supplemental Table 3).

In summary, our analysis of available RNA-seq data from Mateus et al. [1] suggests their conclusions are not based on robust evidence. Most *R. irregularis* genes proposed to be involved in mating by the authors do not show evidence of regulation across conditions and/or at statistically significant thresholds. Most *R. irregularis* genes expected to be involved in mating are not expressed when strains co-exist. The study by Mateus et al. [1] concludes, for example, that: "AMF genes known to be involved in different stages of mating responses in other fungi are upregulated when two genetically distinct strains co-exist in roots", or that "the discovery of in planta activation of genes related to different stages of mating in *R. irregularis* and also provides some clues to understanding the early steps of the evolution of sex-determination of fungal systems".

Although the hypothesis that AMF could mate in planta is intriguing, it is not supported by our re-analysis of the data set from Mateus et al. [1]. First, the above-mentioned statements are not supported by orthology predictions. These predictions are especially important to highlight gene function when members of very large families (protein kinases, HMG) are studied, as virtually any relative of such families shares, by definition, some level of sequence homology. Our re-analysis of RNA-seq data from Mateus et al. [1] also failed to detect significant regulation of *R. irregularis* mating genes during strain co-existence. Specifically, although some evidence of regulation can be found for a few genes in one condition at an adjusted *p*-value of 0.1, the use of conventional statistical standards (adjusted *p*-value of 0.05) and replication (gene regulation must be shared across biological replicates conditions) revealed regulation of a mere two putative *R. irregularis* orthologues of known fungal mating genes. These two genes encode for one putative RNA helicase (out of 21 identified in Rhiir2 gene repertoire) and one velvet factor (out of 6). These genes are part of families involved in a myriad of cellular functions that are not linked to mating [20]. Given the strong emphasis of Mateus et al. [1] on the regulation of mating-related genes, it is surprising that the authors did not investigate the expression of AMF MSG, as these are specifically upregulated during fungal mating. Within this context, our re-analysis found no evidence for their regulation, providing an independent absence of evidence for the presence of sexual reproduction during co-inoculation with strains DAOM197198 and B1.

Overall, the absence of upregulation in mating-related genes in Mateus et al. [1] could be easily explained by the actual incompatibility (as defined, for example, by their divergent MAT- locus sequences [7]) between the strains DAOM197198 and B1 used by the authors. However, identifying the putative compatibility of these two strains is currently unfeasible because the genome of strain B1 has not been sequenced by Mateus et al. [1], despite the fact that each strain may theoretically differ by up to 50% in gene content [8]. Obtaining the genome of the B1 strain would ensure that standard requisites for conventional in silico gene expression analyses are met. Specifically, it would ensure that mapping of RNA-seq reads is performed on the proper reference genome and that the relative transcriptomic contribution of each strain is clearly identified.

Acknowledgements We thank the editor, anonymous reviewers, as well as Vasilis Kokkoris, Jeanne Ropars, and Ricardo. Vega, Toni Gabaldon, Jason Slot, Stefano Ghignone, and the Bioinformatics facilities of the LRSV in Toulouse for their comments on an earlier version of this manuscript. We also thank Jeanne Ropars for providing key analyses for this study. N.C. research is funded by the discovery program of the Natural Sciences and Engineering Research Council (RGPIN-2020-05643) and the Discovery Accelerator Supplements Program (RGPAS-2020-00033). N.C. is a University of Ottawa Research Chair in Microbial Genomics.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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