

Discovery of psychoactive plant and mushroom alkaloids in behavior-modifying fungal cicada pathogens

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This Supplementary Materials PDF file includes:

Supplementary Results

Figs. S1 to S7

References

Links and captions for Tables S1-S17

Supplementary Results

Morphological studies

Morphological studies were conducted to permit species-level identification of collected specimens. Conidia (n = 37) and resting spore (n = 14) measurements were acquired for freshly collected *M. cicadina* (*Mc*)- and *M. platypediae* (*Mp*)-infected cicadas, as well as from archived *M. levispora* (*Ml*)-infected *Okanagana rimosa*, *M. cicadina* and *M. platypediae* (table S2). Morphology of conidia were studied in 15, 14, and 8 specimens of *M. cicadina*, *M. platypediae*, and *M. levispora*, respectively, while morphology of resting spores was examined in 12, 1 and 1 specimens (table S2). The conidial specimens of *M. cicadina*-infected periodical cicadas spanned ten Broods and six of the seven *Magicicada* species collected over 38 years and distributed across the eastern U.S., while *M. platypediae*- and *M. levispora*-infected cicadas were from a single populations collected in New Mexico (2017) and Michigan (1998), USA, respectively (table S2).

Mean conidia (*Mc* = 14.76 μm x 16.65 μm and *Ml* = 8.78 μm x 14.26 μm) and resting spore (*Mc* = 42.42 μm and *Ml* = 40.7 μm) dimensions for *M. cicadina* and *M. levispora* overlapped with previously reported measurements (fig. S1). In contrast, spore dimensions in the majority of *M. platypediae* specimens fell completely outside the reported range for this species (table S2). Interestingly, all the *M. platypediae* measurements fell within the reported range for *M. levispora*, including a single resting spore specimen, which had not been previously reported for *M. platypediae* (fig. S1, table S2).

Molecular and Phylogenetic studies

Molecular and phylogenetic studies were conducted to complement morphological studies and help provide species-level identification of collected specimens. *Massospora* spp., prior to this study, were represented in NCBI Genbank by four DNA sequences from a single isolate of *M. cicadina* (ARSEF 374; NCBI Genbank accessions EF392377, EF392548, EF392492, DQ177436). GenBank blastn results of LSU sequences from *M. cicadina*, *M. levispora*, and *M. platypediae* DNA templates were 86-88% identical to *Eryniopsis caroliniana* strain ARSEF 640 (EF392387), but only 82-84% identical to *Massospora cicadina* strain ARSEF 374. Independent DNA extraction and sequencing of ARSEF 374 fungal plug tissue failed to generate a high

quality LSU sequence and therefore it was not included in the LSU phylogeny. GenBank blastn results of SSU sequences from *M. cicadina*, *M. levispora*, and *M. platypediae* DNA templates were 96-97% identical to *Entomophthora schizophora* (AF052402) and *Entomophthora thripidum* (AF296755). Despite a deposited SSU reference sequence for *M. cicadina*, none of the *Massospora* species sequenced in this study had a reportable BLAST hit to *M. cicadina* ARSEF 374. In contrast, successful amplification of SSU from the resampled ARSEF 374 specimen by our lab did yield a usable sequence 100% identical to other *M. cicadina* strains from this study using pairwise BLAST searches and 97% identical to strains of *E. schizophora* and *E. thripidum*. GenBank blastn results of EFL sequences, an elongation factor like protein present in some early diverging fungi (*I*), from *M. levispora* and *M. platypediae* DNA templates were both 83% identical to *Entomophthora muscae* isolate AFTOL-ID 28 (DQ275343), while *M. cicadina* was 81% identical to *Geranomyces variabilis* voucher JEL379 (HQ901615). Attempts were made to extract and amplify DNA from type specimens of *M. tettigatis* (53297), *M. levispora* (53294), *M. diceroproctae* (53305), and *M. cicadetae* (CUP-060126) but were unsuccessful using Sanger sequencing.

Phylogenetic analysis of the individual partitions (not shown) and combined dataset (LSU+SSU) using maximum likelihood (ML) resolved all sampled *Massospora* in a strongly supported monophyletic group sister to a clade containing *Entomophthora* and *Arthropaga* (fig. S2). EFL was excluded from the concatenated dataset on account of its punctate distribution across the Entomophthorales (*I*). *Massospora cicadina* formed a single lineage that encompassed all sampled broods and *Magicicada* species with the exception of previously deposited *M. cicadina* ARSEF 374, which aligned with *Entomophthora*. Surprisingly, *Massospora levispora* and *M. platypediae* were not resolved as genealogically exclusive species. Instead, they grouped together in a single monophyletic clade clonal lineage sister to *M. cicadina* (fig. S2). Coupled with the morphological data, we propose two possible scenarios: 1) a single annual-cicada infecting *Massospora* species, which includes *M. levispora* and *M. platypediae*, encompasses a broader geographic and host range than previously reported, or 2) *M. levispora*, in addition to *M. platypediae*, can infect wing-banger cicadas. Given that more data is needed to support these hypotheses, each of the three *Massospora* species, *M. cicadina*, *M. platypediae*, and *M. levispora*, will be maintained for clarity.

ML bootstrap analysis of the individual LSU and SSU partitions (data not shown) revealed strong support for two *Massospora* species although a polytomy in the SSU tree could not fully resolve the relationships among *Massospora* spp., a clade containing *Entomophthora* and a fourth clade containing *Arthropaga myriapodina* and *Eryniopsis ptycopterea*.

Reference LSU, SSU, and EFL DNA sequences for *M. cicadina*, *M. platypediae*, and *M. levispora* were deposited in NCBI Genbank (MH483015 - MH483011 and MH547099).

Other alkaloids detected using global metabolomics

Using global metabolomics, other tryptamine-containing natural products were identified from metabolomics databases for both *M. cicadina* and *M. platypediae* at significantly higher levels than the cicada controls. In *M. platypediae*, two lysergic acid pathway product intermediates, 4-Dimethylallyl-L-tryptophan (4-DMAT) and agroclavine (ergot alkaloid), were observed at 17.6x and 16.8x log fold change higher than in *M. cicadina* and in the cicada control, respectively. In *M. cicadina* and *M. platypediae*, aspidospermine, another tryptamine-containing alkaloid, was 16.8x and 14.5x log fold change higher compared to the cicada control. Additionally, Nb-lignoceroyltryptamine was also recovered from *M. cicadina* and was 16.8x log fold change higher than *M. platypediae* and the negative control. Slaframine, a piperidine alkaloid, was also recovered from *M. cicadina* at 13.6x log fold change increase over *M. platypediae* and the control. However, unlike cathinone, psilocybin, and psilocin, none of these other alkaloids were confirmed via fragmentation. Furthermore, analysis using LC/UV for agroclavine failed to confirm its presence. Together these results indicate that these IDs are incorrect and resulted from isobaric compounds (having the same m/z as a previously characterized compound).

Detection of Cathinone from *M. cicadina* resting spores

Results from a second global metabolomics study only comparing resting spore versus conidial isolates of *M. cicadina* showed a more complex metabolome from resting spore isolates (fig S4). Of the 213 metabolites shown to be significantly different between conidia and resting spores ($p < 0.05$) and have a log fold change > 2.5 , only 31 metabolites were shown to be upregulated in the conidia, including cathinone which showed a 4.61 ± 0.28 higher fold change compared to its resting spore counterpart. However, none of the cathinone pathway intermediates

were detected. Further studies are needed to clarify the role of cathinone in secondary resting spore infections.

Detection of Cathinone CoA Dependent Pathway intermediates

Over 10 CoA species were monitored including all five hypothesized cathinone CoA-dependent pathway intermediates, Trans-cinnamoyl-CoA, 3-Hydroxy-3-phenylpropionyl-CoA, 3-oxy-3-phenylpropionyl-CoA, benzoyl-CoA. The most abundant CoAs detected were Acetyl-CoA and Malonyl-CoA with other CoAs detected with varying intensities across pooled samples. However, none of the cathinone CoA-dependent pathway intermediates monitored were detected, meaning they were not present or below detectable limits. Given the labile nature of amphetamines (2) and the nearly 2-year delay in assessing these samples for these pathway specific intermediates, additional freshly collected samples should help clarify these findings.

LC-MS/MS absolute quantification of psilocin

Following the detection of psilocybin from *M. platyediae* using global metabolomics, we sought to quantify this compound from individual fungal plug extracts from all three cicada-fungal pairs including *M. levispora*-infected annual cicadas, which was shown to from a single monophyletic clonal lineage with *M. platyediae*. *Massospora levispora* was excluded from global metabolomics studies due to long-term storage of specimens in ethanol that prevented proper normalization but was still suitable for absolute quantitation. Despite its detection in all *M. platyediae* conidial specimens (table S1), attempts to quantify psilocybin revealed it was below limit of quantification. Given these results we next sought to quantify psilocin, the biologically active form of psilocybin, as this metabolite can be obtained by dephosphorylation of natural psilocybin under strongly acidic or under alkaline conditions. After finding quantifiable amounts of psilocin from *M. platyediae*-infected annual cicadas, we monitored for both compounds in the remaining samples.

LC-MS/MS absolute quantification of monoamine alkaloids from healthy cicadas

To confirm the fungal origin of monoamine alkaloids from *Massospora*-infected cicadas, we performed targeted LC-MS/MS absolute quantification of cathinone, psilocybin and psilocin on independent populations of the two cicada hosts included in the global metabolomics study, *Platypedia putnami* from CA and Brood VII *Magicicada septendecim* from NY. Sampling included five outwardly asymptomatic males and females for each cicada species. Similar to cicada controls used in the global metabolomics, results of these studies failed to detect any of the three monitored compounds from macerated posterior abdominal sections (table S4).

PCR for *Psilocybe* Psi genes

Deposited nucleotide sequences for four psilocybin biosynthesis enzymes (3), PsiD (1-tryptophan decarboxylases, PsiK (4-hydroxytryptamine kinase), PsiM (norbaeocystin methyltransferase), and PsiH (a monooxygenase), described from hallucinogenic mushrooms, *Psilocybe cubensis* and *Psilocybe cyanescens*, were used to develop eight degenerate primer sets in attempt to amplify *Psilocybe* psilocybin biosynthesis genes in *Massospora platypediae*. *Psilocybe cubense* genomic DNA served as a positive control in all PCR reactions. All primer sets failed to amplify Psi-specific PCR targets in any of the assayed *Massospora* DNA templates but three primer sets, one for PsiD, PsiM and PsiK, each yielded amplicons of expected size for the *Psilocybe cubense* control, validating the primer design against psilocybin biosynthesis genes. GenBank blastn results of PsiD, PsiM and PsiK sequences from *Psilocybe* DNA templates were 98-99% identical to *Psilocybe cubensis* strain FSU 12409 (KY984101, KY984100 and KY984099, respectively). All three sequences were deposited in NCBI Genbank (MH483012 - MH483014).

Conidiobolus coronatus pathogenicity assays for alkaloid detection

To test functionality of this single locus uncovered in *Conidiobolus coronatus* (Entomophthorales) during queries for evidence of psilocybin gene clustering among homologs of the candidate sequences, eight representative *C. coronatus* isolates spanning two insect orders (Hemiptera and Thysanoptera), Basidiomycotan fungi, and a horse (table S12) were acquired from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures

(ARSEF) chosen for live-plating and injection entomopathogenicity assays on *Galleria mellonella* (4). By 24-hours post-inoculation, all inoculated individuals were dead and by 48-hours all live plated individuals were symptomatic with many dead larvae. Symptomatic individuals were collected at 48-hour post-inoculation and immediately placed at -80 C prior to maceration to permit targeted LC-MS/MS absolute quantification of psilocybin and psilocin targeted metabolomics from both infected larvae and pure cultures of each isolate. Results of these studies failed to detect either compound from macerated infected larvae or week-old fungal cultures of these same isolates on Sabouraud Dextrose Yeast Agar. However, *C. coronatus* isolate ARSEF 8715 (NRRL 28638), from which the genome and candidate locus was obtained, was not available and therefore was not included in the study.

Parsimonious reconstructions of active host transmission in Entomophthoralean fungi

Any topology that resolves the clade containing *Massospora* and *Entomophthora* sister to *Strongwellsea* requires either two independent gains of active transmission behavior, one of which is followed by a single loss, or a single gain in the ancestral progenitor followed by three losses, the latter of which suggests this behavior modification may have been the key innovation that led to diversification of the Entomophthoralean fungi. Active host transmission is known from four of some 20 Entomophthoralean genera (5,6), two of which, *Entomophthora* and *Entomphaga*, also include well known examples of summit behavior. Presumed delays in *Massospora*-induced mortality require further confirmation as do their cause(s) which may include changes in: 1) behavioral heliothermy, the basic thermoregulatory pattern in cicadas (7) which may restrict pathogen growth inside the host (8); 2) pathogen virulence including the expansion or contraction of hydrolytic enzymes, such as chitinases, proteases and lipases (9); 3) other extrinsic biotic and abiotic factors (8); or 4) a combination of these factors.

Female-typical courtship behaviors by male *Massospora*-infected *P. putnami* and the possible hormonal changes driving these behaviors

Activity-level effects of psilocybin may provide a plausible explanation of the addition of female-typical behaviors to the repertoires of male *Massospora*-infected wing-banger cicadas.

Infected males were found to produce significantly fewer crepitations (wing tapping required for mating) per observation period (10), a female-typical behavior, possibly resulting from impaired temporal processing, a known effect of psilocybin in vertebrates (11). However, the atypical mating behaviors of other cicada species may be better explained by *Massospora*-mediated hormonal changes incidental to amphetamine and psilocybin production (fig. S5). For example, 2-Hydroxyestradiol, a catechol estrogen and metabolite of estradiol and 3-O-acetylcodynone 2-phosphate, a metabolite of ecdysone, a steroidal prohormone of the major insect molting hormone 20-hydroxyecdysone, were both found to be upregulated in male *M. platypediae* compared to a mixed sex group of *M. cicadina* and healthy controls using global metabolomics. These hormones may accumulate as a result of disruption of typical pathways (i.e. castration of testis) or they may be fungus-derived.

Testing for facultative plant associations in *M. cicadina*

The root colonizing properties of *Massospora* on sudangrass (*Sorghum sudanense*), a model system for arbuscular mycorrhizal fungi (<https://invam.wvu.edu/methods/cultures/host-plant-choices>) were assessed following the discovery of a plant-based metabolite, cathinone, in *Massospora*. Ten plants were inoculated with 300 *M. cicadina* resting spores, identical to inoculations using AMF spores. After five months, plants were harvested and roots washed to confirm whether root colonization had taken place. Root staining of four randomly chosen plants failed to confirm a facultative mutualism with plants.

Supplementary Figures

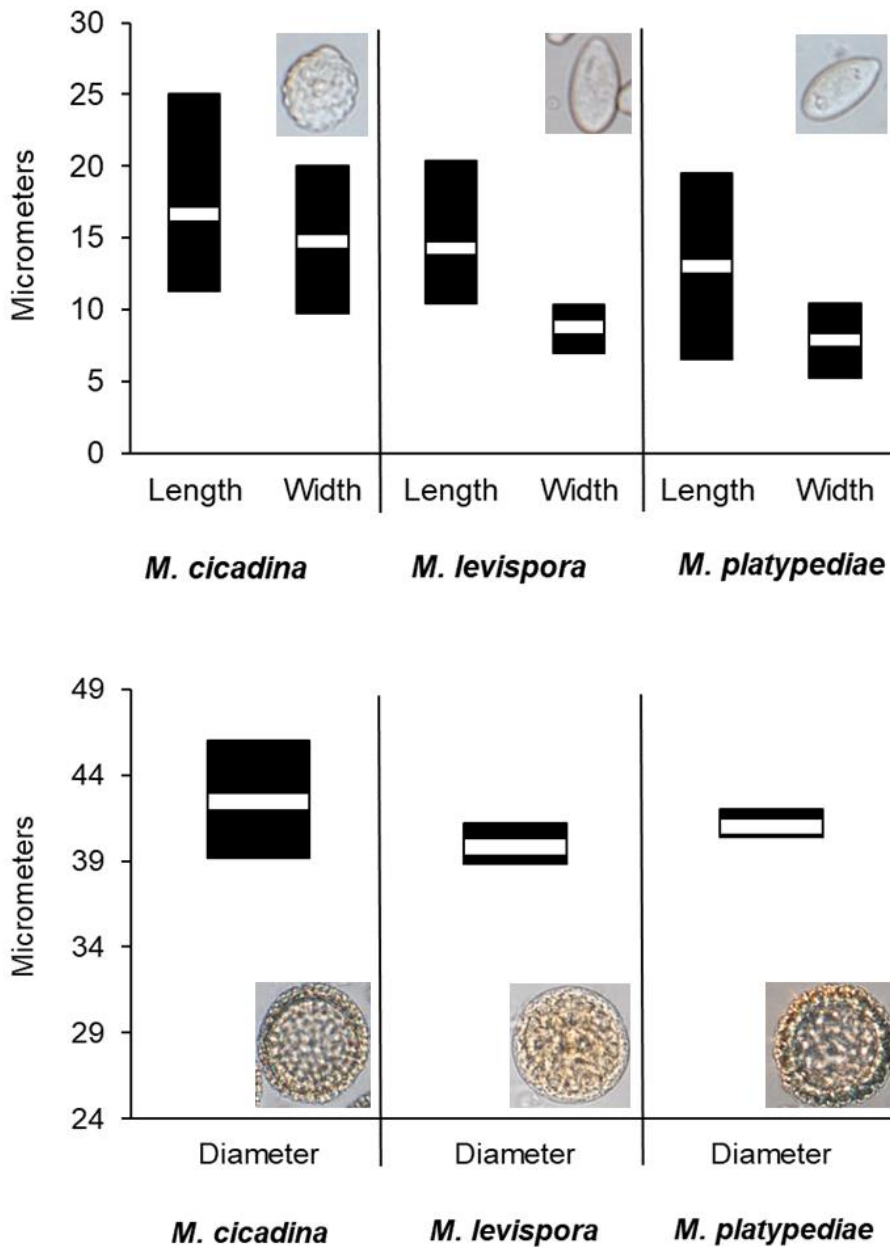


Fig. S1. Mean (A) conidia and (B) resting spore dimensions for three *Massospora* species sampled from infected cicadas. White bars indicate overall mean values while black bars indicate the range of variation in spore dimensions. Twenty-five conidia or resting spores were measured for each specimen except for *M. levispora* and *M. platypediae* resting spores, in which 50 spores were measured. Sampling sizes were as follows: For conidia $M_c = 15$, $M_l = 8$, and $M_p = 14$; For resting spores, $M_c = 11$, $M_l = 1$, $M_p = 1$.

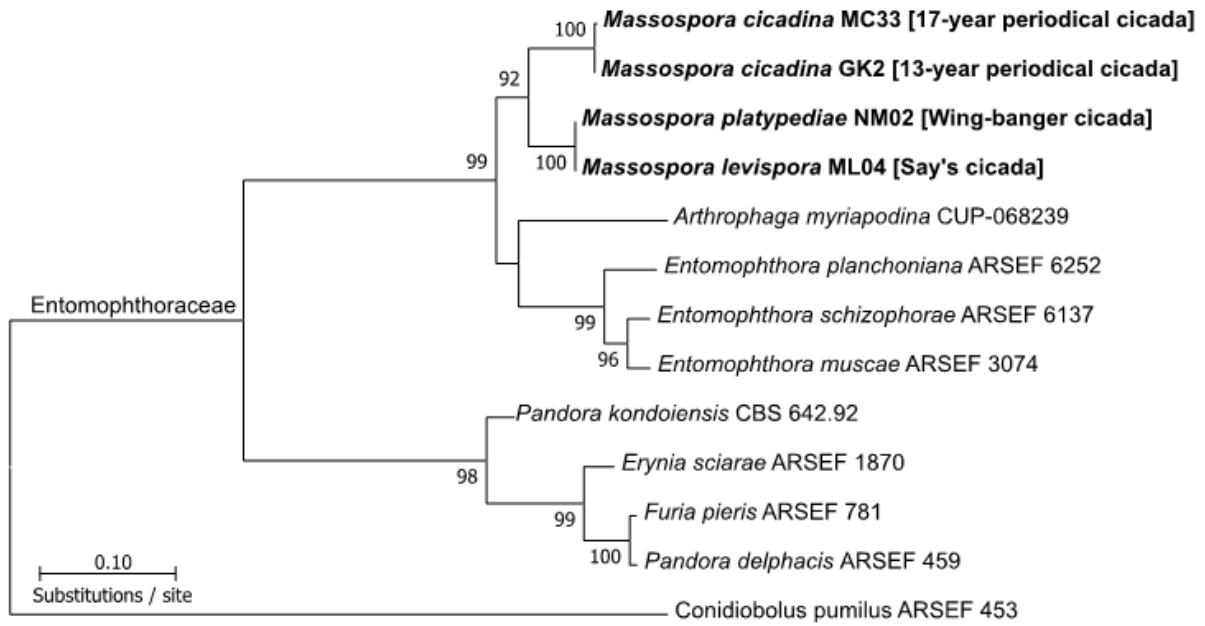


Fig. S2. Concatenated LSU+SSU maximum likelihood (ML) tree consisting of *Massospora* species and related species in the Entomophthorales. Bootstrap support is indicated near each node and only values greater than 70% are shown.

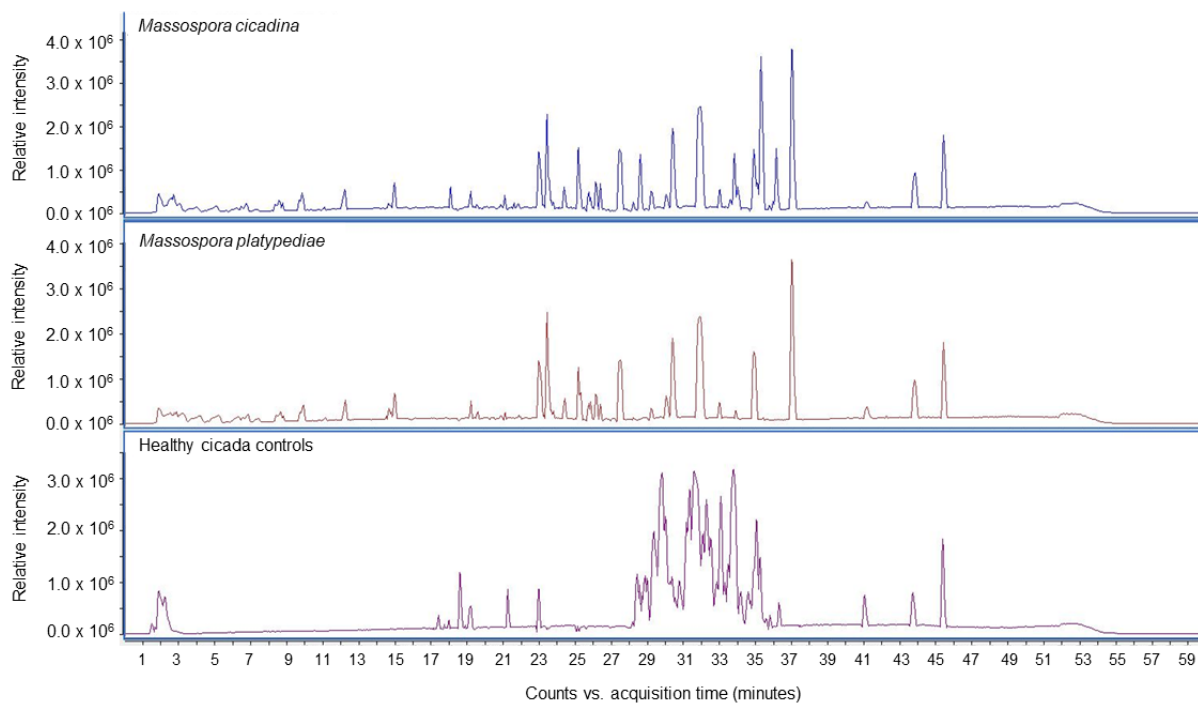


Fig. S3. Representative total base peak chromatograms (BPC) from the global metabolomics analysis of *M. cicadina*, *M. platyepidae*, and a healthy cicada controls.

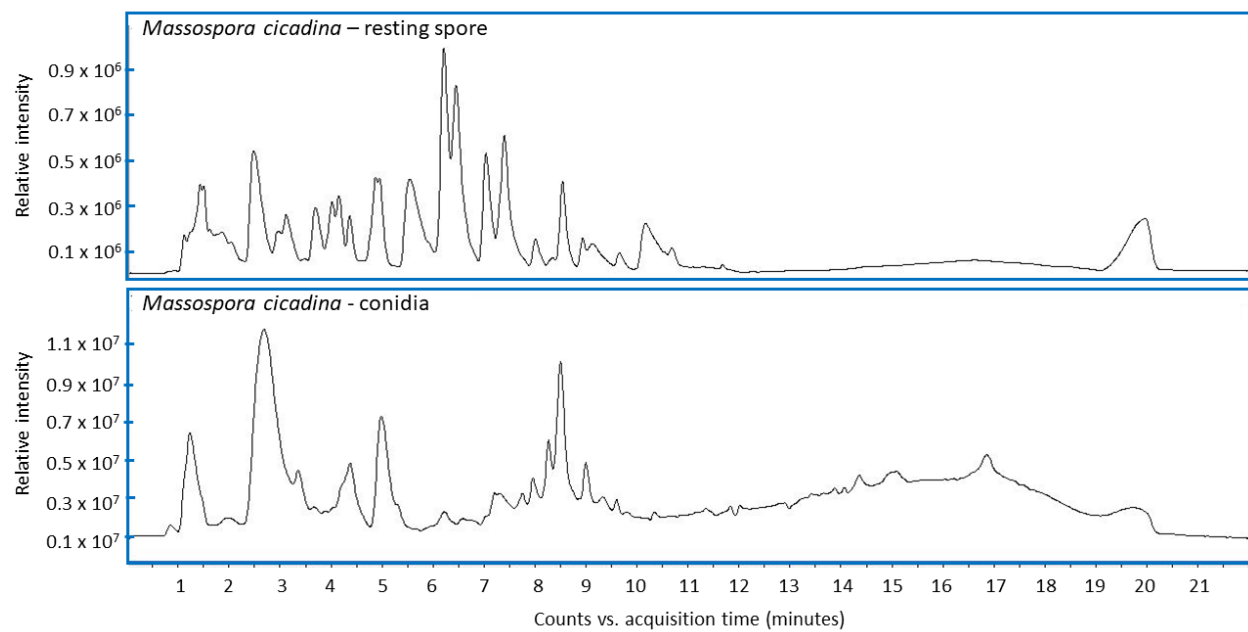
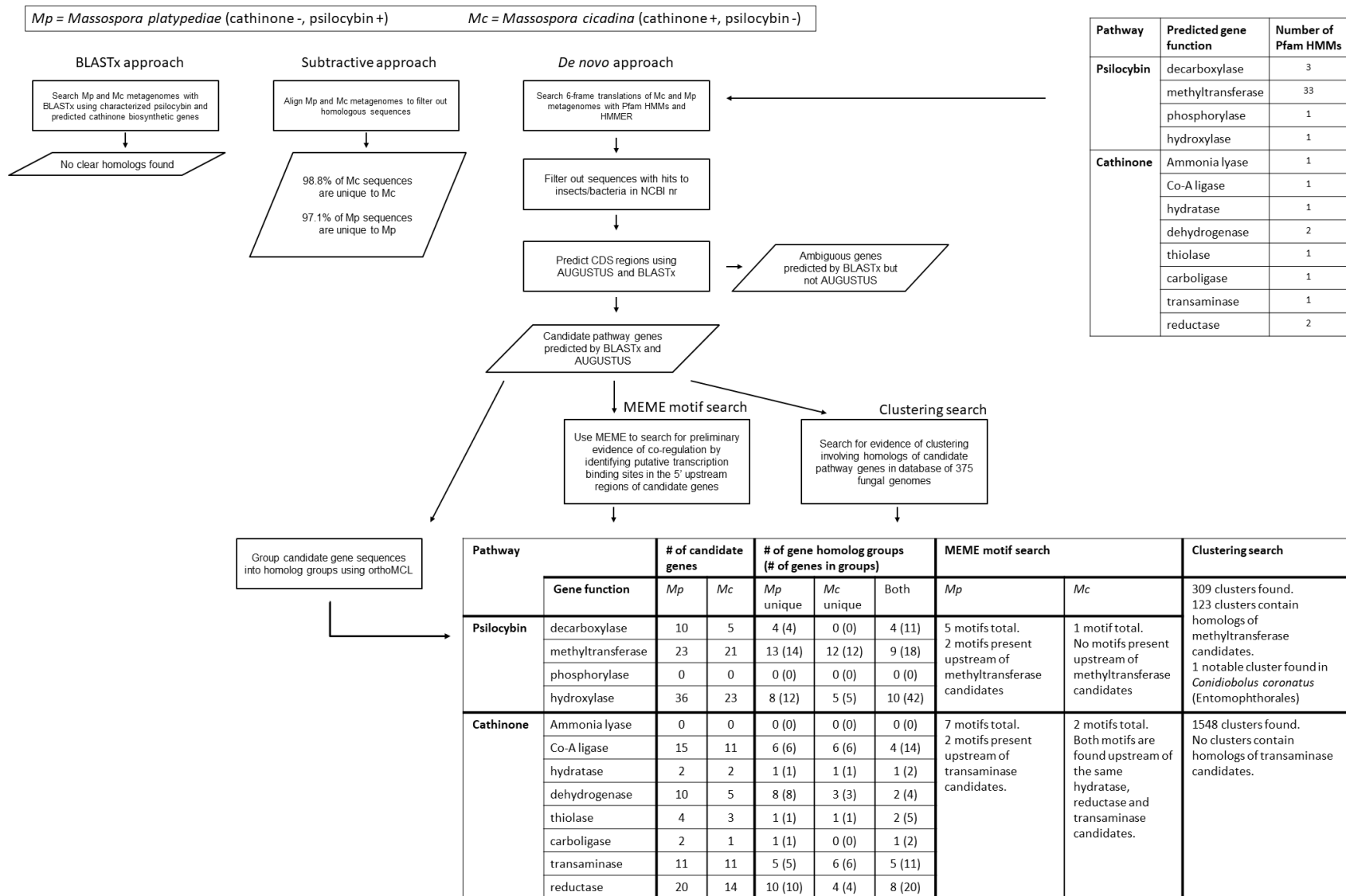
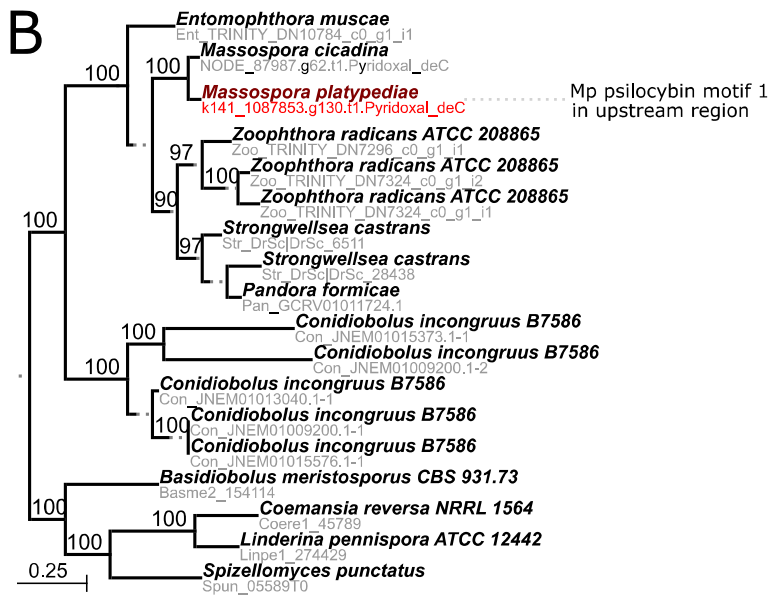
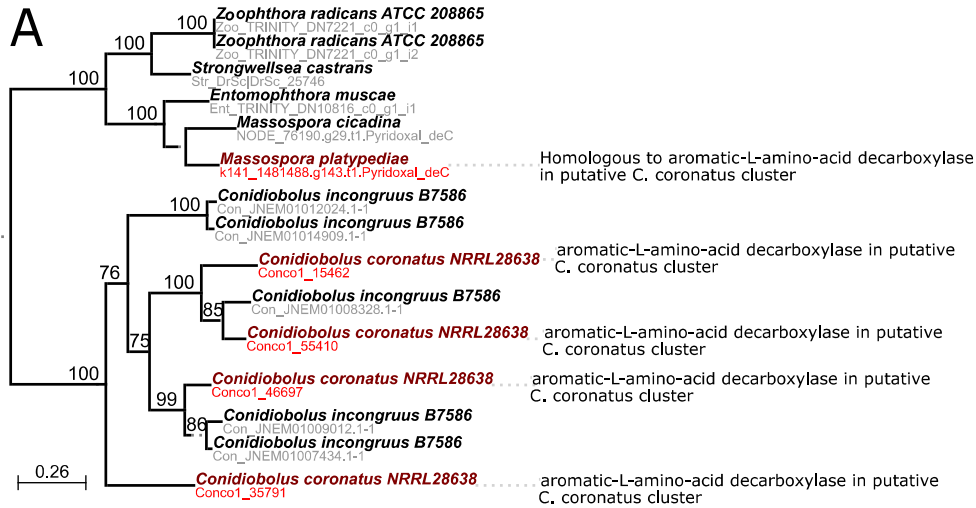


Fig. S4. Representative total base peak chromatograms (BPC) from the global metabolomics analysis of *M. cicadina* resting spores versus *M. cicadina* conidia.



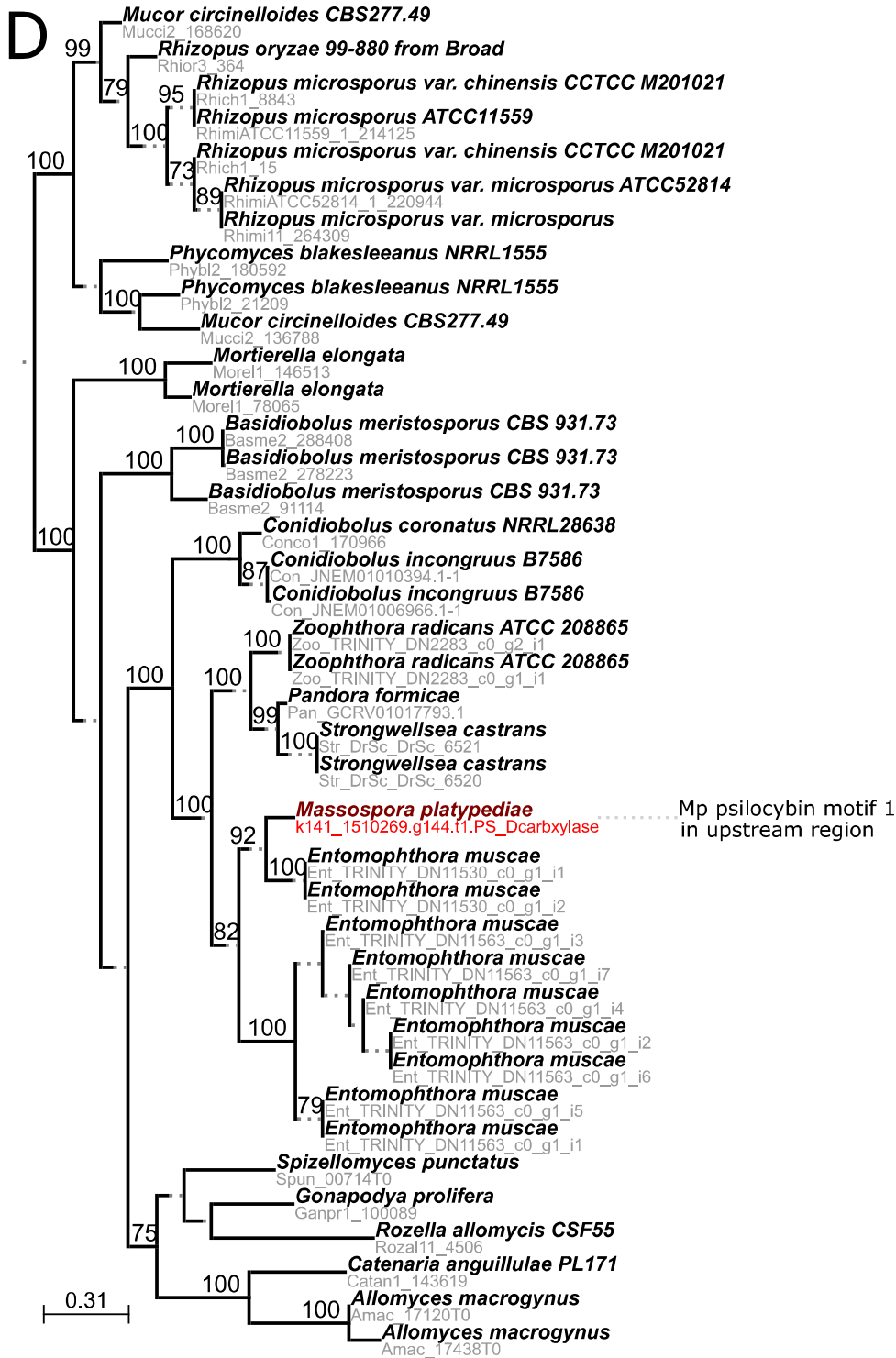
Pathway	Predicted gene function	Number of Pfam HMMs
Psilocybin	decarboxylase	3
	methyltransferase	33
	phosphorylase	1
	hydroxylase	1
Cathinone	Ammonia lyase	1
	Co-A ligase	1
	hydratase	1
	dehydrogenase	2
	thiolase	1
	carboligase	1
	transaminase	1
reductase	2	

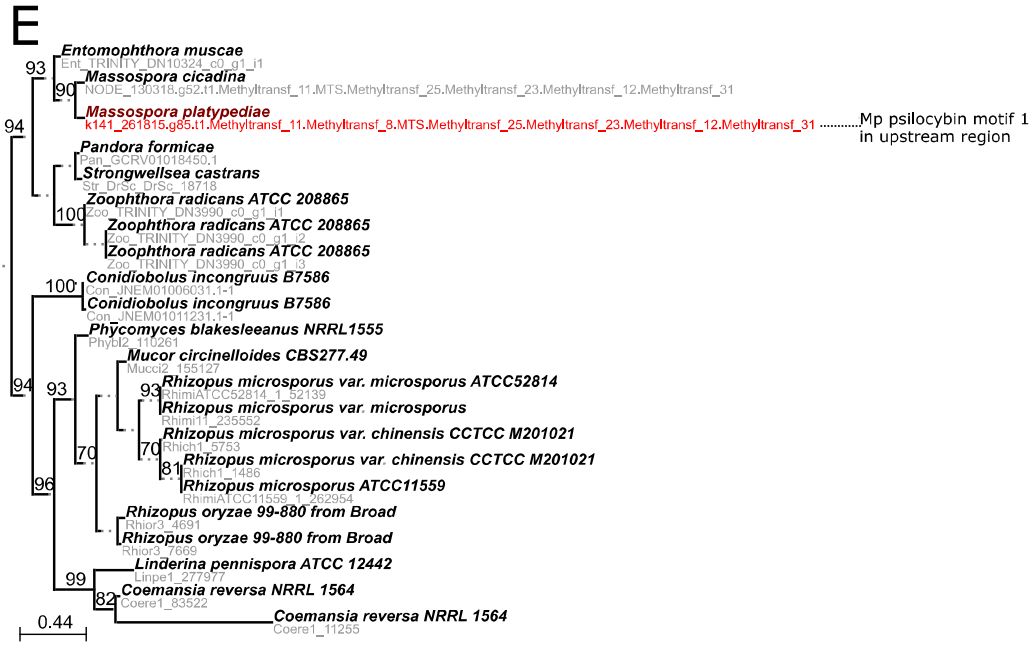
Fig. S5. Psilocybin and Cathinone biosynthesis pathway gene discovery pipeline.

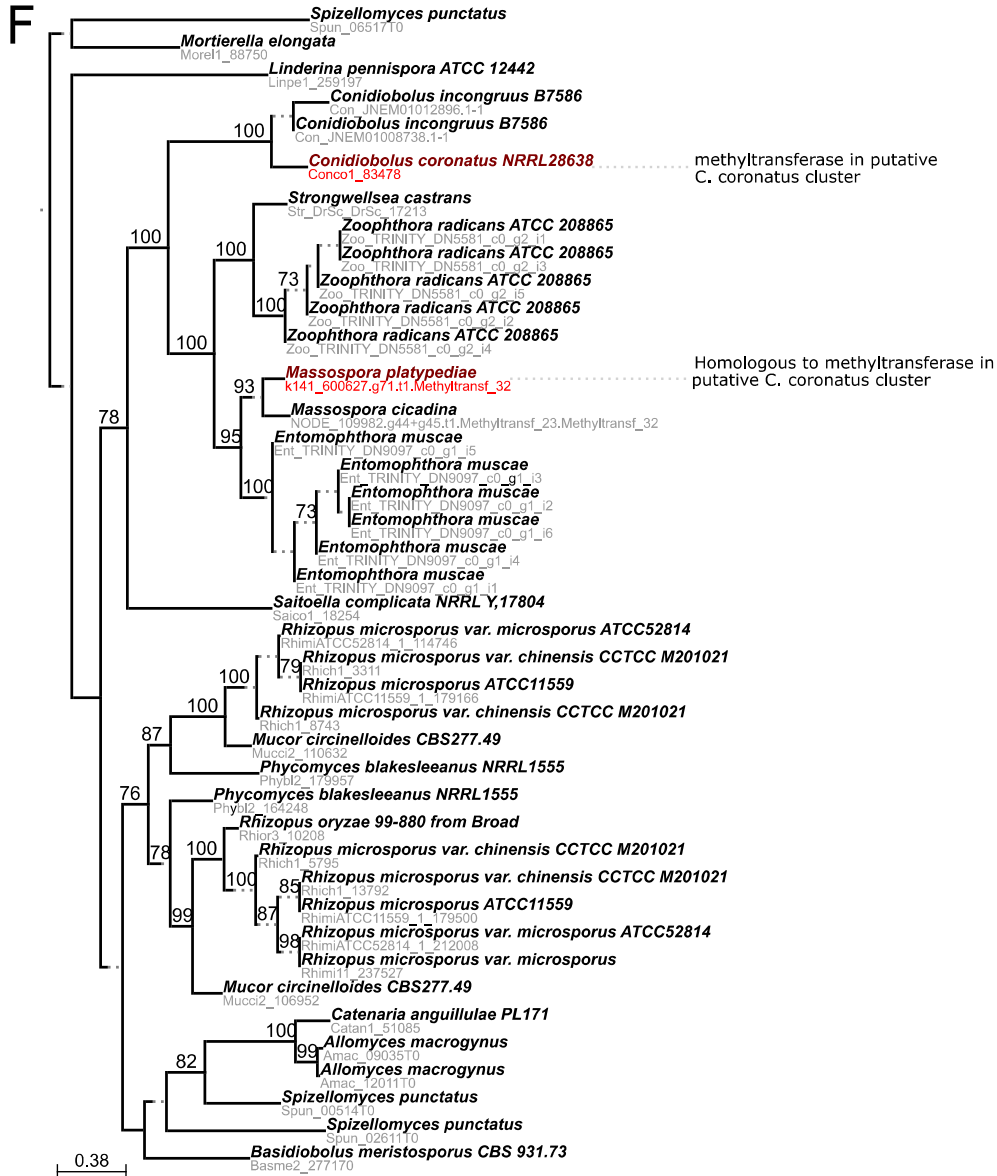


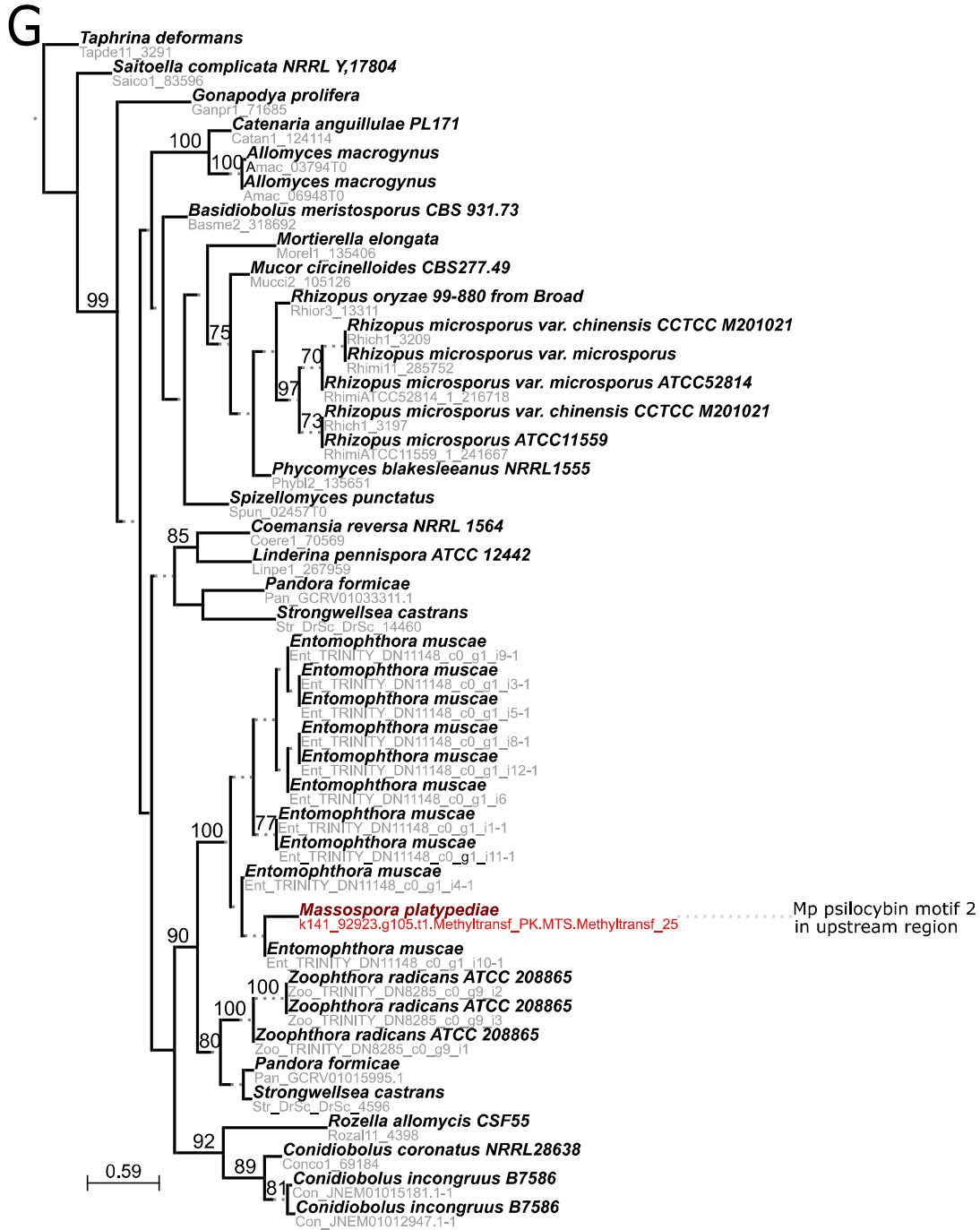
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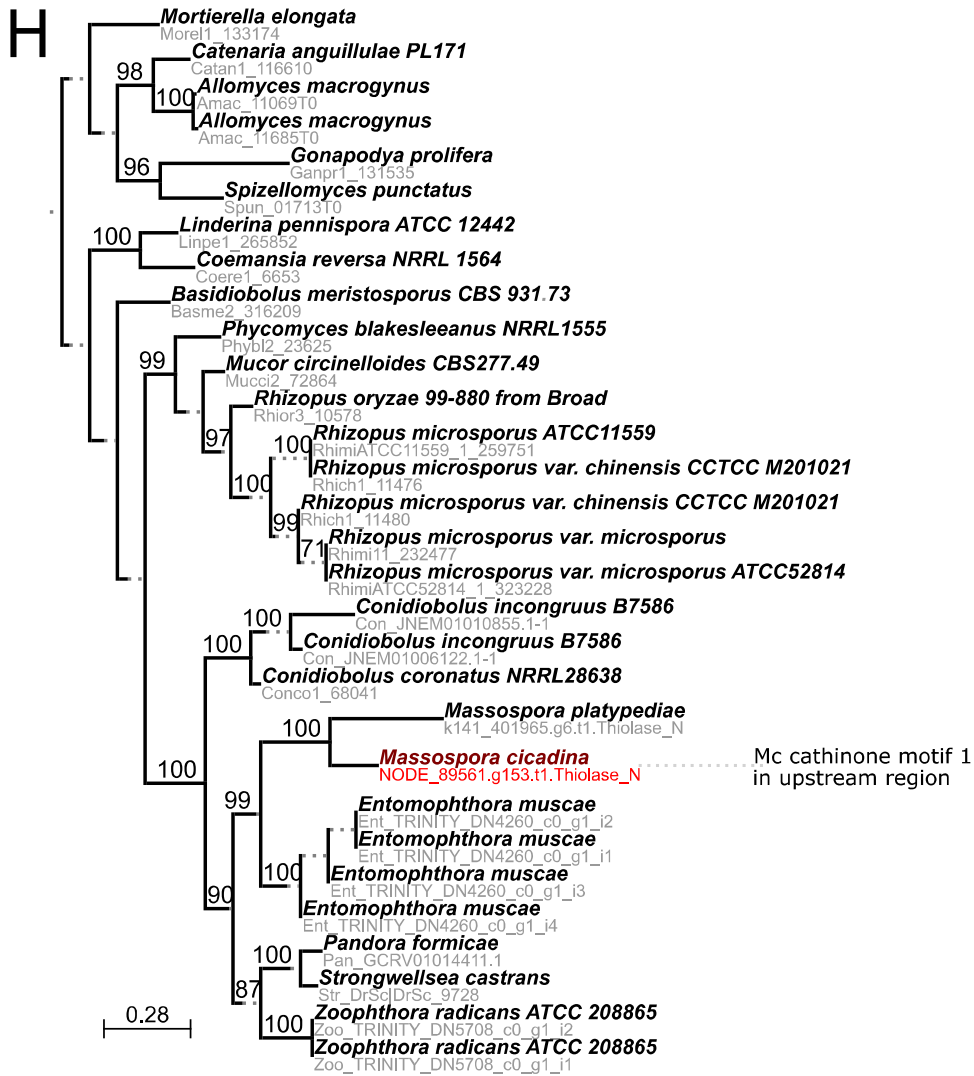


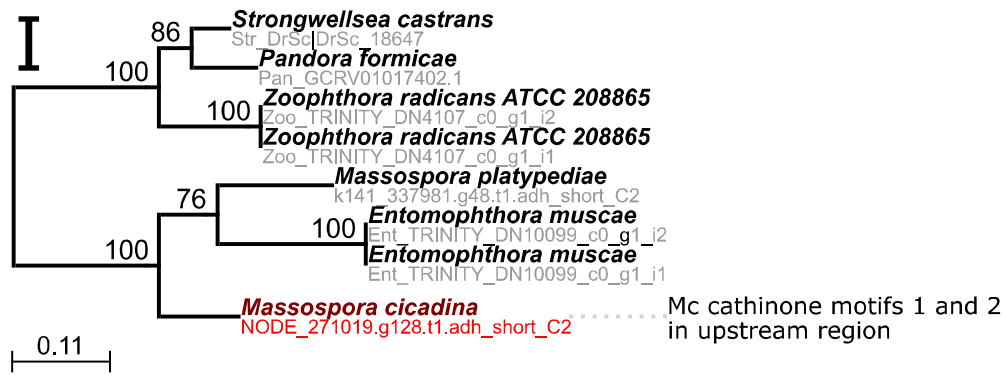


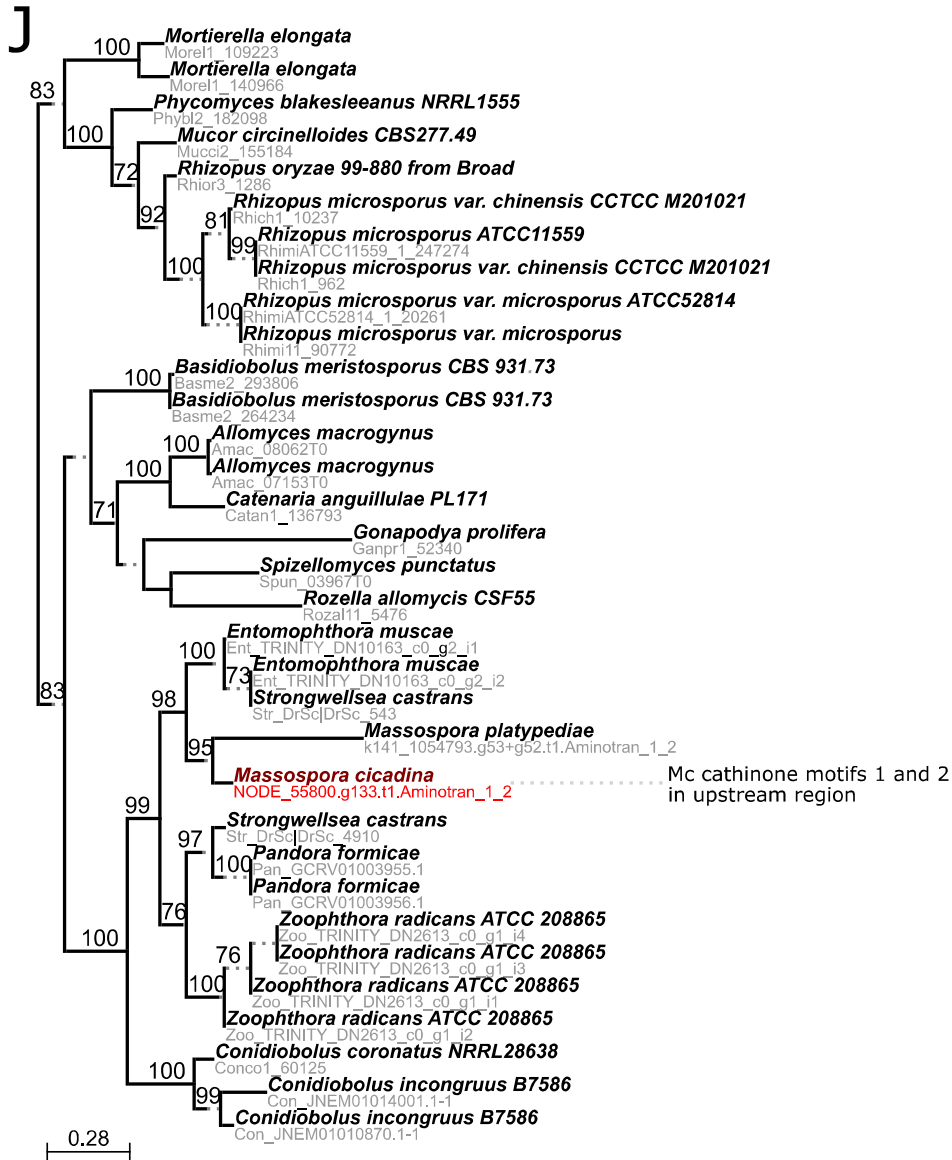




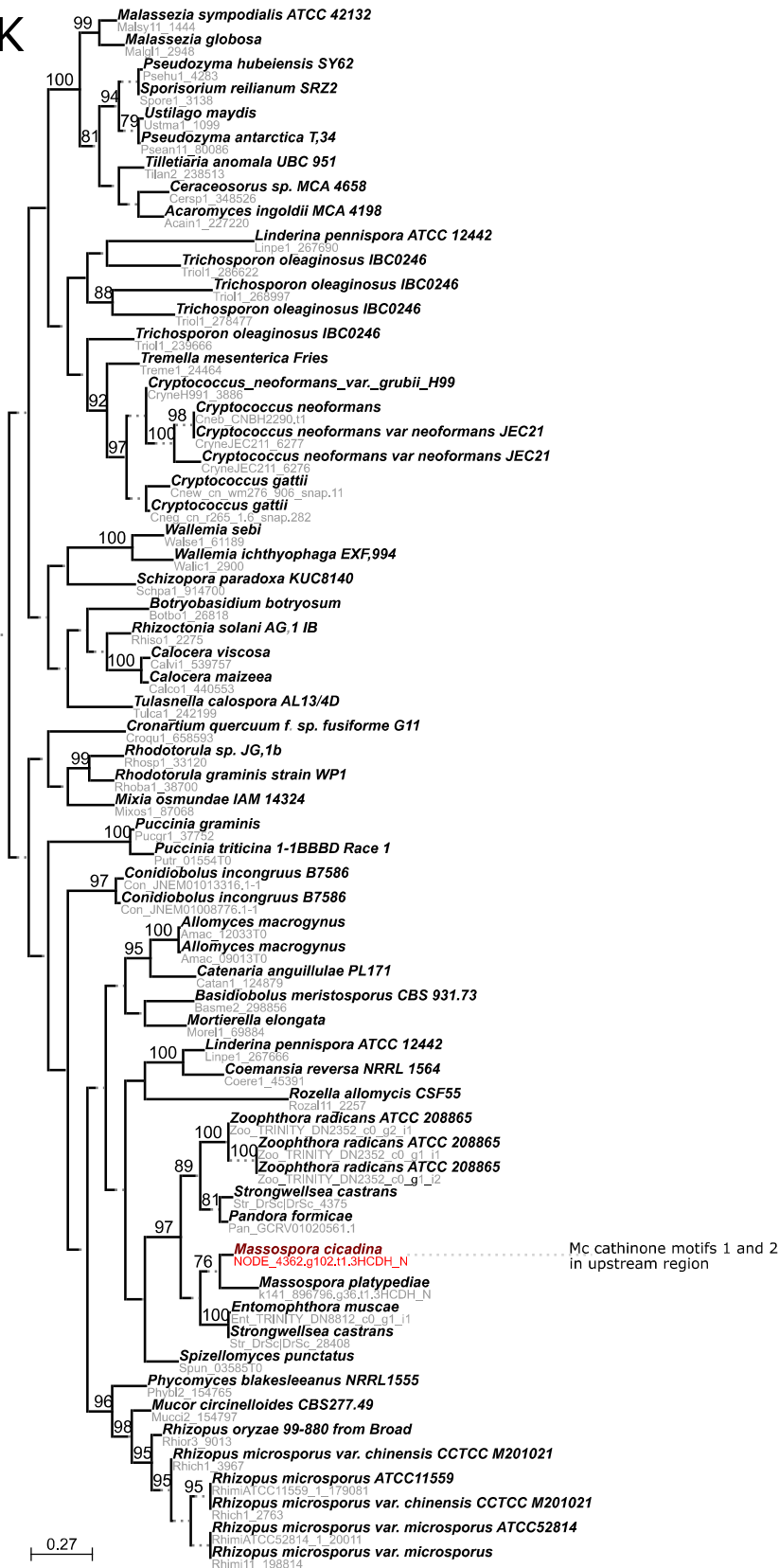


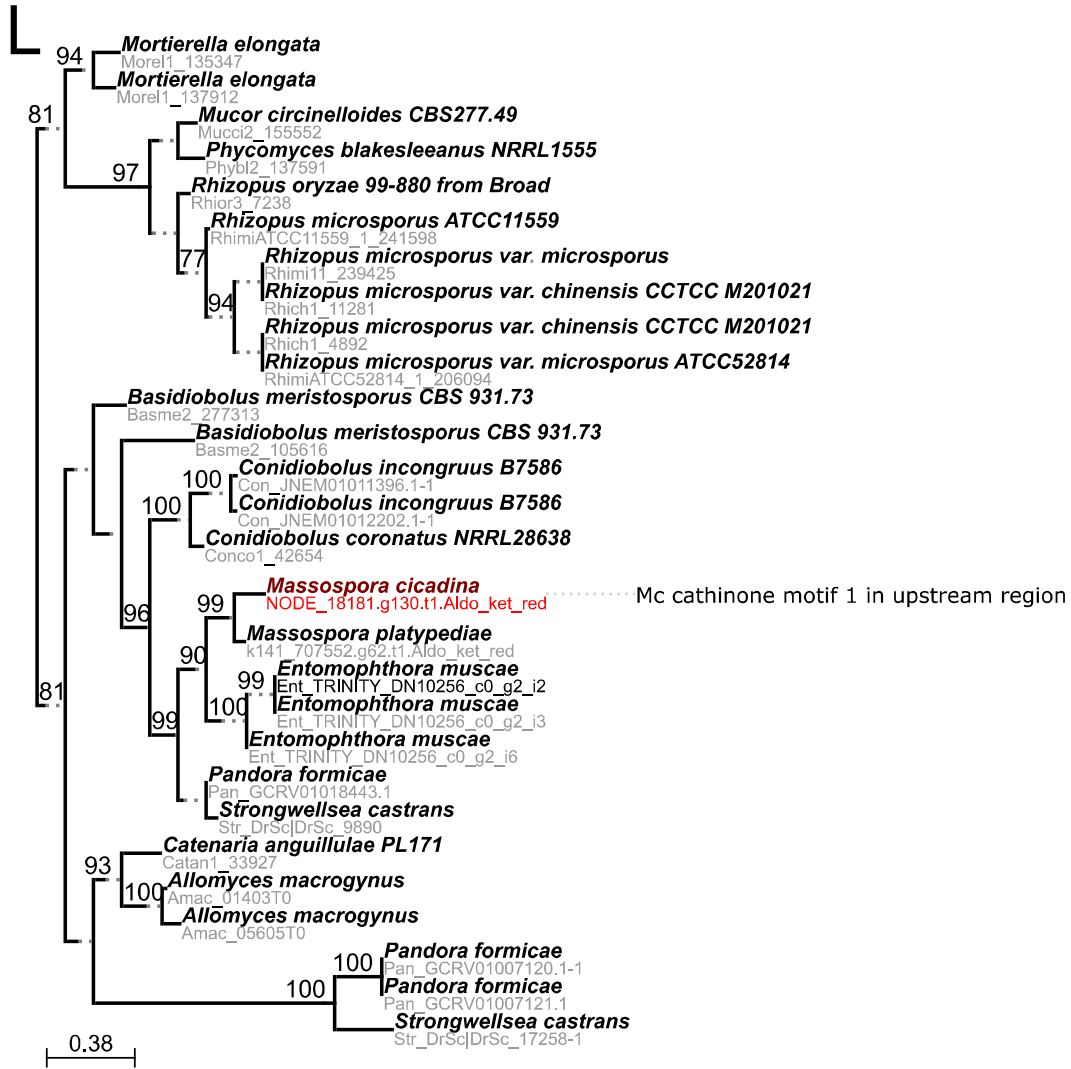


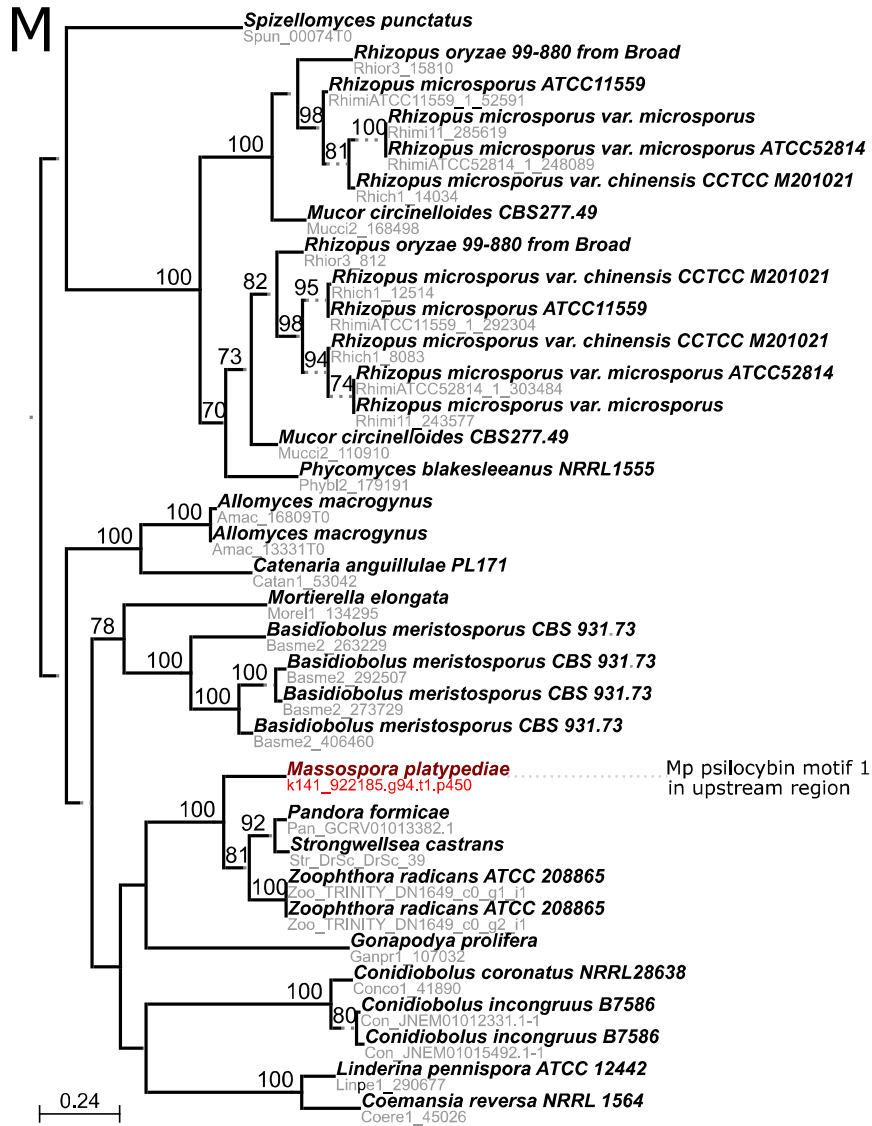




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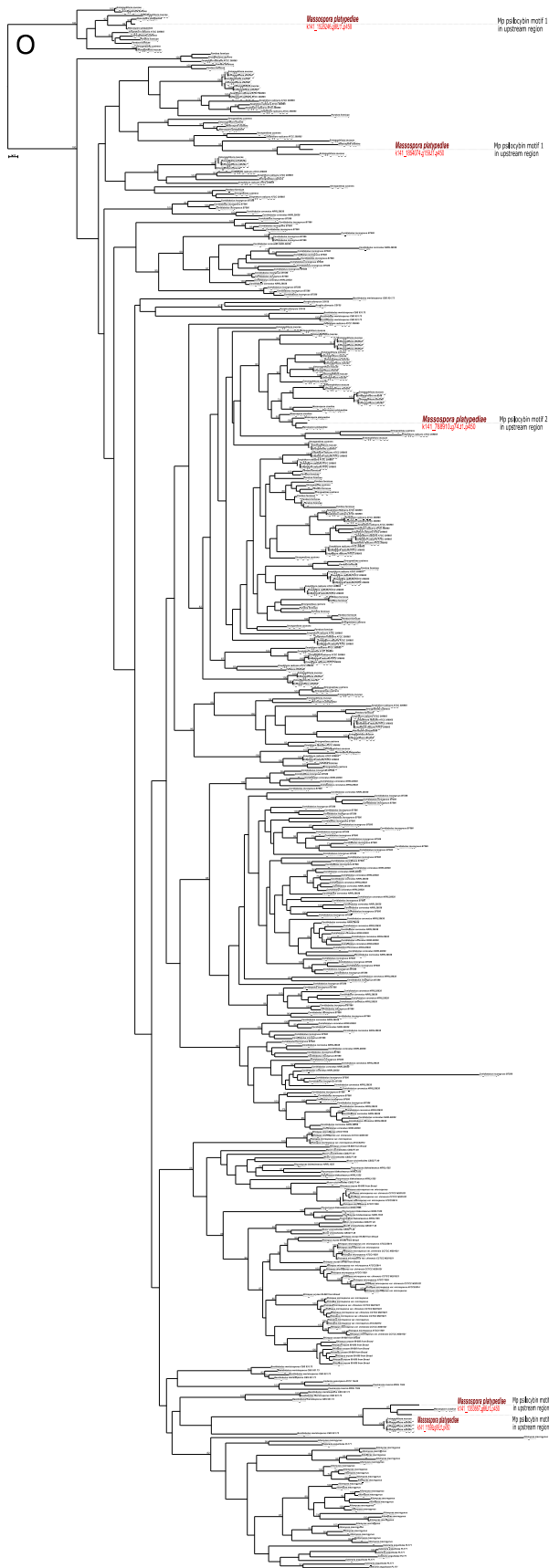






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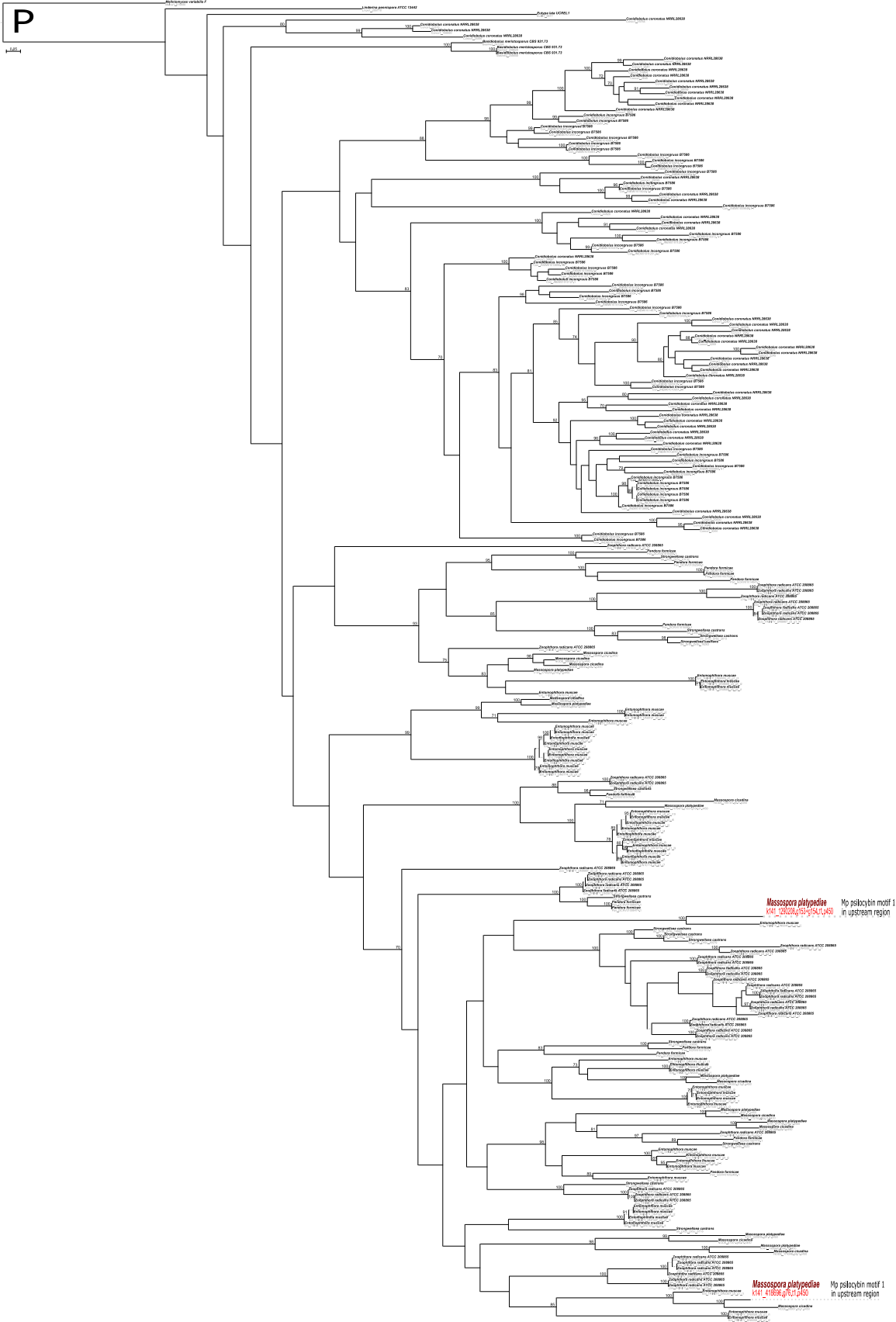


Fig. S6. Candidate ML gene phylogenies potentially involved in cathinone and psilocybin biosynthesis pathway from *M. cicadina* and *M. platypediae*. Phylogenies include (A-C) three Group II pyridoxal-dependent decarboxylases (Pyridoxal_deC), (D) one Phosphatidylserine decarboxylase (PS_Dcarbxyase), (E-G) three methyltransferases (Methyltransf), (H) one acetyl-coenzyme A acetyltransferase (Thiolase_N), (I) one Enoyl-(Acyl carrier protein) reductase (adh_short_C2), (J) one Aminotransferase class I and II (Aminotran_1_2), (K) one 3-hydroxyacyl-CoA dehydrogenase, NAD binding domain (3HCDH_N), (L) one Aldo-keto reductase (Aldo_ket_red), (M-P) ten Cytochrome P450 (p450).

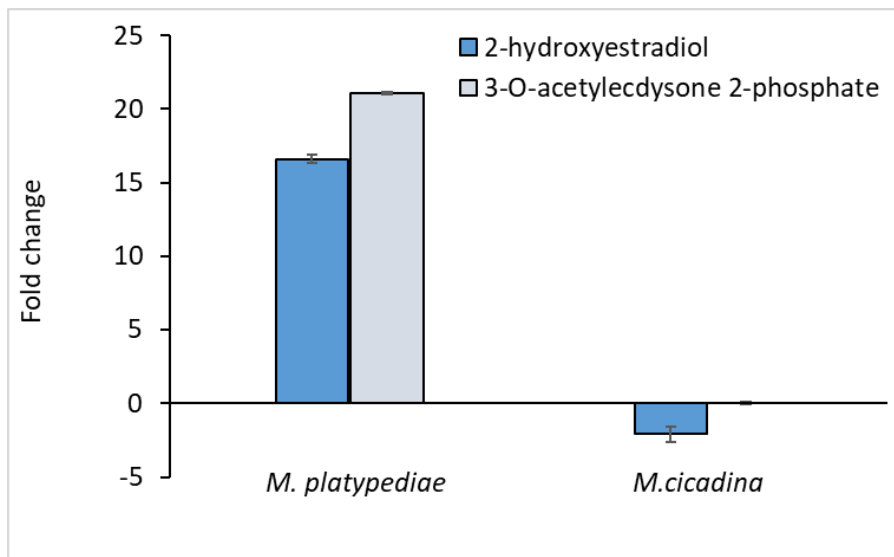


Fig. S7. Differential fold change and regulation of 2-Hydroxyestradiol and 3-O-acetylcyclohexane 2-phosphate expression in conidial plugs of *M. platypediae* and *M. cicadina* using global metabolomics. The former metabolite is an endogenous steroid and the latter is a product of ecdysone, a steroidal prohormone of the major insect molting hormone 20-hydroxyecdysone.

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Table S1-S17. All data available at: [10.6084/m9.figshare.6854951](https://doi.org/10.6084/m9.figshare.6854951)

Table Legends:

Table S1. Changes in cicada behavior as a result of *Massospora* infection compared to outwardly healthy counterparts.

Table S2. *Massospora* spore measurements. Conidial and resting spore measurements base on the average of 25 measured spores per fungal isolate except for *M levispora* and *M. platypediae* resting spore measurements, which were based on 50 spores. Underlined mean values in parentheses denote that mean length and width fell within the previously reported ranges for that given species.

Table S3. Confirmation of Cathinone, psilocybin, and their pathway intermediates from pooled *Massospora* conidial plugs using MS/MS fragmentation. †Fragmentation patterns for cathinone, psilocybin, and psilocin based on fragmentation of commercially available analytical standard. Fragmentation patterns for all other pathway intermediates based on published literature.

Table S4. Quantification of psilocybin, psilocin, and cathinone from *Massospora* fungal plugs against commercially available, DEA-exempt analytical standards using targeted LC-MS. Samples that fell outside the limit of quantification were scored as either present or absent only. ND, not detected; BLQ, below limit of quantification; * U.S. state abbreviation, † indicates samples were included in two independent runs, ‡ isolates used in global metabolomics studies, § isolate from which metagenome was sequenced.

Table S5. All profile HMMs used to retrieve candidate genes.

Table S6. Homolog groups of candidate cathinone biosynthetic genes

Table S7. Homolog groups of candidate psilocybin biosynthetic genes

Table S8. Metadata for all genomes part of local database

Table S9. All motifs detected in the 1500bp 5' upstream regions of candidate *Massospora* genes. Cells highlighted in beige indicate a motif present upstream of a predicted transaminase while cells highlighted in purple indicate a motif present upstream of predicted methyltransferase.

Table S10. clusters containing a methyltransferase retrieved from database with 375 genomes

Table S11. Annotation of genomic region surrounding the notable *Conidiobolus coronatus* cluster containing homologs to candidate psilocybin biosynthetic genes. Cells highlighted in beige indicate a protein homologous to candidate psilocybin gene from *Massospora*.

Table S12. *Conidiobolus coronatus* strains used in targeted metabolomics studies alone in culture and inoculated into the greater wax moth (*Galleria mellonella*).

Table S13. High quality candidate genes for psilocybin and cathinone biosynthesis

Table S14. Initial FASTTREE phylogenies of high quality candidate genes

Table S15. Proteins recovered from whole protein extraction of *Massospora cicadina*.

Table S16. Strain histories for genome-wide phylogeny.

Table S17. Primer sequences used for amplification of *Psilocybe* psilocybin (Psi) biosynthetic genes. Target = *Psilocybe* spp. psilocybin (Psi) biosynthetic genes, Amp = amplicon size, T = annealing temperature, Ext. = extension time, and GenBank = NCBI Genbank accession numbers.