The Invisible Engine of Cymbidium goeringii Nutritional Growth - Mycorrhizal Symbiosis

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Abstract

More than 80% of all terrestrial plant species are symbiotically associated with mycorrhizal fungi. This study revealed that the orchid endomycorrhiza and pine ectomycorrhiza have similar symbiotic culture. We addressed the long-standing question that whether the two investigated fungus are belong to completely different symbiotic groups or reflect the same species in different hosts with different symbionts. Mycorrhizal fungi were isolated from the photosynthetic orchid *Cymbidium goeringii* and identified as members of Ceratobasidiaceae by metagene method and phylogenetic analysis of the internal transcribed spacer (ITS) region of ribosomal DNA. It is an ectomycorrhizal fungus formed by *Pinus yunnanensis*. The ITS sequences were similar among geographically separated samples obtained from Golden Temple National Park in Kunming city of Yunnan province. The isolated fungus, KMGT47, formed ectomycorrhiza on seedlings of *Pinus yunnanensis* in pot culture. This suggest that tripartite symbiosis exists between *C. goeringii*, mycorrhizal fungi, and *P. yunnananses* in nature, and carbon compounds could be supplied from *P. yunnananses* to *C. goeringii* through the hyphae of the mycorrhizal fungi. To the best of authors' knowledge, this is the first study the suggest the involvement of Ceratobasidiaceae fungi in tripartite symbiosis with photosynthetic orchids and photosynthetic host plants. It was verified by metagene and polygenic phylogenetic analysis.

Introduction

Orchids are among the most prized ornamental plants in many societies throughout the world. The genus *Cymbidium* includes over 50 species of terrestrial, lithophytic, and epiphytic orchids from mountainous regions of China, Korea, Japan, Southeast Asia, and India (Llamas,2003). Which include *C. goeringii* as one of the species types. *C. goeringii* is a photosynthetic orchid, which is distributed from Yunnan province in China to Korea, Japan and Southeast Asia (DuPuy & Cribb, 1988). This orchid is usually found at the understory of coniferous or broad-leaved forests. In China, the orchid flowers bloom around early December to late February, with fructification and seed dispersal occur in a sequence within 12 months.

Clarification of mycorrhizal symbiosis in *C. goeringii* would contribute to better understanding the life cycle of photosynthetic orchids. However, to the best of authors' knowledge, no studies have been conducted to identify the mycorrhizal fungi of this orchid. The symbioses with ectomycorrhizal fungi have been reported for many types of achlorophyllous orchids such as *Cephalanthera austinae* with Thelephoraceae (Taylor & Bruns, 1997), *Hexalectris spicata* with Sebacinaceae (Taylor et al., 2003), and *Corallorhiza maculata* and *C. mertensiana* with Russulaceae (Taylor & Bruns, 1999). In those cases, the mycorrhizal fungi were identified from fungal DNA sequences. Furthermore, tripartite symbiosis was confirmed in several achlorophyllous orchid *Chamaegastrodia sikokiana* species as well (Takahiro Yagame, 2008). The artificial cultivation from seed germination to flowering in *Rhizanthella gardneri*, so-called underground orchid, was achieved under symbiosis with a mycorrhizal fungus isolated from *R. gardneri* rhizome and *Melaleuca uncinata* host tree (Warcup, 1985). In the *C. trifida* species, the development of hyphal links of mycorrhizal fungi was observed from host trees, *Betula pendula* or *Salix*

repens, and carbon transfer was detected from the host trees to the investigated orchid (McKendrick et al., 2000).

The *C*. *goeringii* was usually found at the understory of ectomycorrhiza-forming trees, such as *P*. *yunnanensis*, and endomycorrhizal roots are often found around the rhizomes of this orchid. Therefore, it was hypothesized that this orchid might have symbiotic relationship with some ectomycorrhizal fungi. Determining the microorganisms in a complex community is one of the great challenges. With the development of sequencing technology, Metagenomics is becoming an important method to investigate microbial communities, regardless of their culturability (Cowan et al., 2005), which can detect of microbial diversity and prediction of functional genes (Ranjan et al., 2016). The variation of mycorrhizal fungal community composition among different orchid was obvious. Some strains are un-culturable and the exact diversity and abundance of mycorrhizal fungi in *C*. *goeringii* may be underestimated. The main aim of this study was to isolate and identify the mycorrhizal fungi of *C*. *goeringii*, in order to form an ectomycorrhiza on *P. yunnanensis*. It was verified by metagene and polygenic phylogenetic analysis.

Materials and methods

Plant Growth and Treatment

The seeds and mycorrhizae of *Cymbidium goeringii* were collected from Golden Temple National Forest Park Conservation and the Gaoligong Mountains Conservation Station in Yunnan province. This action was approved by the Management Bureau of Golden Temple National Forest Park Conservation and the Gaoligong Mountains Conservation Station. The seed collection of *C. goeringii* in the Gaoligong Mountains was permitted by the private landowners. The seeds of *C. goeringii* were cultivated in the tissue culture room of the Biodiversity Key Laboratory at Southwest Forestry University. After germination, the seedlings were transplanted into separate plastic pots with a diameter of 12 cm and a height of 10 cm, using a sterile substrate mixture of sand, vermiculite, and mica in a 1:1:1 ratio, and grown in a cultivation room where temperature, humidity, and light intensity could be automatically controlled. According to actual conditions, the seedlings were watered approximately every 5 days, and after 3 months of cultivation, a symbiotic cultivation experiment was conducted. The tripartite symbiotic cultivation experiment was carried out in the smart greenhouse at Southwest Forestry University. These seedlings were identified by Professor Deng lilan, a taxonomy Professor at Southwest Forestry University. The identified specimens have been stored in the Herbarium of Southwest Forestry University, with the number LXL-003.

Orchid collection and fungal isolation

To obtain natural mycorrhizal fungal strains from *Cymbidium* plants, the *C*. *goeringii* rhizomes were collected from two forests within approximately 503 km distance from each other. The first sampling site was a coniferous forest of *P*. *yunnananses* in Golden Temple National Forest Park Conservation

(August 2011 and 2012) 2076 m above sea level and located on Kunming City, Yunnan province, China. The second sampling site was an evergreen broad-leaved forest of Quercus acuta and *Pinus yunnanensis* Franch (July 2010) 1980 m above sea level and located at GaoLiGong Mountain, Baoshan City, Yunnan province, China (Fig. 1a, b).

Mycorrhizal fungi were isolated from the collected rhizomes according to Warcup and Talbot (1967) with slight modifications as follows. The rhizome surface was washed with tap water and sterilized by immersing in 70% ethanol for 1 min and then in sodium hypochlorite solution, containing 1% available chlorine, for 1 min. A piece of the sterilized rhizome, approximately 5 mm in length, was then put into 1 mL of sterilized distilled water in a Petri dish (9 cm in diameter) and crushed with a sterilized glass rod to disperse intracellular hyphal coils (pelotons). About 20 mL of autoclaved Modified Melin-Norkrans medium (MMN, containing 10.0 g glucose, 1.0 g $[CH(OH)\ COONH_4]_2$, 0.5 g KH_2PO_4, 0.15 g $MgSO_4{\cdot}7H_2O$, 0.05 g $CaCl_2{\cdot}2H_2O$, 1.2 mg $FeCl_3$, 0.1 mg thiamine hydrochloride, 3.0 g malt extract; 2.0 g yeast extract, 10 mL vegetable juice (V8), 15.0 g agar and 1,000 mL distilled water) (Marx, 1969) was cooled down to 45°C and poured into the Petri dish. The sample was then shaken before solidification of the medium to disperse the pelotons and incubated at 25.0 ± 0.5°C in the dark for 3 days. Fungal colonies growing from the pelotons were isolated with a sterilized scalpel.

Ectomycorrhiza formation in pot culture

The *C. goeringii* was found in a forest of *P. yunnananses* in its habitat in Golden Temple National Forest Park. Therefore, this woody plant species was used in the pot experiment to form ectomycorrhiza. The *P. yunnananses* seeds were sown in pots containing the substrates, which were autoclaved at 121°C for 60 min to eliminate any microorganisms in the substrate. In each pot, four *P. yunnananses* seedlings were grown in a greenhouse for 1.5 year before mycelium inoculation. Three fungal strains, KMGT36, KMGT47, BSGLGM28, isolated from a *C. goeringii* rhizome was grown in 150 mL of liquid PDA medium at 25.0 ± 0.5°C in the dark for 1 month, and the mycelium was buried as an inoculum near the roots of *P. yunnananses*. The inoculated seedlings were grown in a greenhouse for another 3 months and the formed ectomycorrhizas were observed during this period.

Molecular identification of isolated fungi

The total DNA from each fungus cultured on PDA medium and ectomycorrhiza formed on *P. yunnanensis* was extracted using the CTAB method (Xu et al., 2000). The internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) was amplified using the polymerase chain reaction (PCR) with primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes & Bruns 1993; White et al., 1990). A typical PCR reaction volume of 25 µl were established, containing 12.5 µL of 2 × *Taq* PCR Master Mix (with Blue Dye), 1 µL of each primer (10 µM), 1 µL DNA template, and 9.5 µL sterilized ultrapure water. The reaction was performed using the following conditions: initial denaturation step at 95°C for 5 min, a subsequent step of 35 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 1 min and

a final elongation step at 72°C for 10 min. The PCR products were cloned using a pT7Blue Perfectly Blunt Cloning Kit (Novagen, Madison,WI) according to the manufacturer's instructions and plasmid DNAs were extracted from the cloned products using MagExtractor Plasmid (TOYOBO). The plasmid inserts were sequenced with the dye terminator method using sequencing primers M13-47 and RV-M. Obtained sequence data were deposited in the DNA Data Bank of NCBI database. All of the sequenced data were subjected to BLAST search and analogous data were downloaded from NCBI/GenBank nucleotide sequence database. The multiple sequence alignments and neighbor-joining (NJ) phylogenetic analysis (Saitou & Nei, 1987) were carried out for the sequenced and the downloaded data, using Clustal X version 1.83 (Thompson et al., 1994). The evolutionary distances were estimated for NJ phylogenetic analysis, using Kimura's two-parameter model (Kimura, 1980) and bootstrap analysis (Felsenstein, 1985) was performed with 1,000 replications. The phylogenic tree was drawn using Treeview (Page, 1996) and the classification of fungi corresponded to that of Kirk et al. (2001).

Extraction of total DNA from wild C. goeringii

The surface disinfection of orchid mycorrhizal samples collected in the field was carried out as follows: cut part of the root samples with sterile scissors, wash the soil and other impurities on the root surface with running tap water, wash with phosphate buffer for 3 times, soak in 75% ethanol for 1–2 min to remove other microorganisms on the root surface, wash with phosphate buffer for 3 times, and finally draw 100 μL of sterile water rinsed for the last time and spread it on PDA and LB medium plates, and invert the culture for 5 days to observe whether there are colonies to grow, so as to verify whether the surface disinfection is thorough.

After surface disinfection, the roots were put into a sterile mortar and quick-frozen with liquid nitrogen and ground to powder, weighed 0.3 g and transferred to a sterile centrifuge tube, and the total DNA of the root system was extracted by magnetic bead method plant genomic DNA extraction kit, each sample was extracted 3 times, the DNA integrity was detected by agarose gel electrophoresis, and the DNA sample concentration was quantitatively detected by Qubit3.0.

Metagenomic sequencing and sequence analysis

A total amount of 1 μg DNA per sample was used as the input material for the metagenomic sequencing. Sequencing libraries were generated following the manufacturer's recommendations, the library preparations were sequenced on an Illumina HiSeq 4000 platform, and paired-end reads were generated. The sequencing method was pair-end 150 bp (PE150). The quality of the raw reads was evaluated using FastQC software, and the quality filtration and contamination removal were performed using Trimmomatic vl.0.36 software. Assembly of metagenomic sequences was performed using MEGAHIT v1.2.4 (Li et al., 2016), and binning sequence assembly was performed using the program SPAdes v3.13.1 (Nurk et al., 2013). Sequence alignment was performed using Bowtie2 v2.3.5.1 (Langmead & Salzberg, 2012). MetaGeneMark v3.25 was used to predict genes for the metagenomic data set (Zhu et al., 2010). Gene annotation was carried out with software DIAMOND v0.9.9.110

(Buchfink et al., 2015) to compare (blastp, evalue ≤ 1.0×10^{-5}) (Feng et al., 2015) the target sequences with each functional database to find the best annotation. The databases used were: NR (https://www.ncbi.nlm.nih.gov/), CAZy v20,150,704, (Cantarel et al., 2009), GO (Ashburner et al., 2000), and KEGG (Kanehisa et al., 2014, 2006).

Results

Fungal isolation and Phylogenetic analyses

The rhizomes were collected from two *C. goeringii* rhizome individuals in Gold Temple conservation and one individual in GaoLiGong Maintain. Fungal colonization was found in rhizomes that were colored brown to black and most cortical cells in the rhizome were colonized by coiled hyphae (Fig. 2). In total, 58 strains of mycorrhizal fungi were isolated from the eight rhizome samples. It was found that the isolated fungi originating from the same rhizome formed apparently identical colonies.

The DNA sequences of the ITS regions were obtained from all 58 isolates. Based on BLASTn search comparisons with sequences from representatives of the broad Rhizoctonia fungi in the GenBank, three representative isolates(KMGT36, KMGT47 and BSGLGM28) were selected for phylogenetic analysis. We identified that these strains had three unique ITS sequences and none of them had an ITS three different methods, all the basal branches and the majority of the terminal branches showed consistent clutering patterns among the sequences. Specifically, for this study, all three phylogenetic analyses consistently clustered our three strains with the same groups of sequences, as dexcribed below. The analyses and comparisons identified that strains KMGT36 and KMGT47 shared an identical ITS sequence, and phylogenetic analysis placed them basal to the AG-D group of the genus *Ceratobasidium*. Their sequence identities to the most closely related AG-D group of sequence ranged between 78.1% (to AG-D-3) and 83% (to AG-D-2). Similarly, the ITS sequences of strain BSGLGM28 were distinctly different from all of the published GenBank sequences, and the strain were most closely clustered with representative sequences were most closely clustered with representative sequences of the AG-G group, also within *Ceratobasidium*. The ITS sequence identities of strain BSGLGM28 to the most closely related AG-G group of sequences were 93.2% (between BSGLGM28 and GenBank accession DQ102410). While our analyses confirmed that these three straind belonged to the broad Rhizoctonia fungi, the high relative sequence divergence between our strains and the sequenceed taxa of Rhizoctonia fungi in the GenBank suggest that these three strains may represent two or three new taxonomic groups. The GenBank accession numbers for our three ITS sequences are FJ211862-FJ211864. Sequence identical to any of the sequences deposited in the GenBank.

Because of the large number of taxa included for the comprehensive analyses shown in supplementary data, for better visualization, we selected a subset of the taxa for further analyses using three different phylogeny construction algorithms: MP (Fig. 3), ML (Fig. S1) and NJ (Fig. S2). Though minor differences were found among the phylogenetic trees generated by the three different methods, all the basal beanches and the majority of the terminal branches showed consistent clustering patterns among the

sequences. Phylogenetic analysis showed that three representative isolates have very similar ITS sequence (99.6–99.9%) and are closely related to *Ceratobasidium* spp. It was also found that orchid mycorrhizal fungi isolated from *C. goeringii* rhizome were related to the isolated fungi (Fig. 3).

Ectomycorrhiza formation

The KMGT36, a mycorrhizal fungus isolated from *C. goeringii* at Golden Temple National Forest Park Conservation region, was used for ectomycorrhiza formation. Five months after fungal inoculation, ectomycorrhiza were formed on the rootlets of *P. yunnanensis* (Fig. 4a, b.) and fungal sheath and Hartig net were found in the ectomycorrhiza (Fig. 4c). The ITS sequence similarity between the formed ectomycorrhiza and the inoculated fungus were high at 99.8–99.9%.

Analysis of gene annotations and function prediction

Metagenome sequencing generated 60,901,956 and 57,719,326 reads of raw data form two orchid mycorrhizal samples. After removal of low-quality reads, 60,187,184 and 57,277,600 reads of valid data (Clean Data) were obtained (Table 1). The total length of the genes in the non- redundant sequences was about 46.35 Mbp, and the average length was 307.26 bp.

Table 1
Data of endophytic microbes of C. goeringii roots by metagenomic sequencing

Sample	Raw Data		Clean Data		Clean/%	Q_{20}/%	Q_{30}/%	GC/%
	Read	Base/G	Read	Base/G				
CGkm	60901956	9.14	60187184	8.72	98.83	98.23	94.18	36.93
CGbs	57719326	8.66	57277600	8.37	99.23	98.33	94.40	38.90

Microbial community structure can be represented by the proportion of each taxa. Based on the integrity of the annotation information, the data at the phylum and family level were selected to analyze species composition. Overall, about 94% of the species are unclassified, 4–5% are Bacteria, 1% are Eukaryota, and 0.1–0.2% are Viruses, and 0.01% of species are Archaea. The dominant phylum are Firmicutes (1.83%-2.27%), Proteobacteria (0.96%-1.54%) and Bacteroidetes (0.77%-1.02%), Basidiomycota (0.40%-0.43%) and Ascomycota (0.31%-0.37%) (Fig. 5a). In Eukaryota, the orchidaceae mycorrhizal fungi that have been widely recognized before is relatively low, and only Ceratobastiaceae fungi are in the top 20 relative abundance (Fig. 5b). In order to further analyze the species composition of endophytic fungi in orchid mycorrhiza, we annotated the species of endophytic fungi. The results showed that the relative abundance of endophytic fungi was 1.14%-1.27%, among which, the dominant families were Glomeraceae (16%-18%), Polyporaceae (13%-15%), Ceratobasidiaceae (7%-8%) and Sclerotiniaceae (7%-8%). 5%-6%) and Mucoraceae (4%-6%) (Fig. 5c). The relative abundances of fungi species in the two samples were similar, and Ceratophoraceae were the main OMF groups in *C. goeringii*. Interestingly,

metagenomic sequencing results showed that Glomeraceae colonized in the mycorrhiza, and Glomeraceae had a high abundance in CGkm sample.

To further analyze the functions of predicted genes by metagenomic sequencing, common functional database annotations for non-redundant gene sets were done using DIAMOND software (e-value ≤ 10 − 5), 42,687 sequences were aligned to the KEGG database (Fig. 6). In the first level, the number of annotated metabolism pathways is the largest, with a total of 26,466. Followed by genetic information processing (5,994); environmental information processing (4 921 articles); cellular processes, 1,966 in total. At the second level of the metabolic pathway, amino acid metabolism, carbohydrate metabolism and energy metabolism are the most annotated pathways. The annotation results of KEGG database revealed that the endophytic microorganisms promoted the growth and development of spring orchid mainly by regulating amino acid and carbohydrate metabolism.

Through the metagenome sequencing of orchid mycorrhizae in the wild, it can be seen that there are mycorrhizal fungi Ceratobasidiaceae and Tulasnellaceae in the mycorrhizae, which are the sexual stage of Rhizoctonia inoculated on sterile tissue culture seedlings, and also the ectomycorrhizae formed by us on *P*. *yunnanensis*, and the orchid and *P*. *yunnanensis* form a triangular network symbiosis through the hyphal network, transporting the nutrients they need (Fig. 3).

Discussion

The mycorrhizal fungi of *C. goeringii* were identified as members of Ceratobasidiaceae. One of the isolated fungi formed ectomycorrhiza on *P. yunnanensis*, suggesting tripartite symbiosis among *C. goeringii*, mycorrhizal fungi, and *P. yunnanensis*. No hyphal growth was observed on the PDA medium, which has been used for isolation of saprobic fungi from orchid mycorrhiza, whereas active hyphal growth was observed on the PDA medium, which was used for ectomycorrhizal fungi. This finding suggests that the isolated cultures from the investigated orchid mycorrhiza were all ectomycorrhizal fungi.

The ITS sequences amplified from the formed ectomycorrhiza have high similarity, 99.8–99.9%, to that of the inoculated fungus, KMGT36, which strongly suggests that ectomycorrhiza was formed by the fungus isolated from *C. goeringii*. The slight difference would be caused by the existence of different sequences among rDNA repeats or artifacts in PCR. Due to high sequence similarity among the mycorrhizal fungi isolated from geographically separated samples, it is suggested that *C. goeringii* may have a strong relationship with this group of fungi. Further extensive study is required to examine the specificity of the relationship, by amplifying the fungal DNA directly from the rhizome or pelotons. A detailed investigation of ectomycorrhiza surrounding the rhizome of *C.goeringii* is also desired to reveal the relationship.

It is well known that many orchid species have symbiotic relationships with *Rhizoctonia* spp. The anamorphic fungi, *Rhizoctonia*, are classified into three families of teleomorph, Tulasnellaceae Sebacinaceae, and Ceratobasidiaceae (Peterson et al. 1998), and orchid mycorrhizal fungi have been

reported in all of the three families. In the Ceratobasidiaceae family, orchid mycorrhizal fungi have been reported in *Ceratobasidium* and *Thanatephorus*, e.g., *Ceratobasidium cornigerum* (Bourdot) Rogers in *Thrixspermum congestum* (F. M. Bail) Dockr. (Roberts, 1999; Takahiro, 2008 and 2012), and *Thanatephorus pennatus* Currah in *Calypso bulbosa* Holzing (Currah, 1987). Generally, fungi that belong to this family are regarded as saprobes. Meanwhile, a mycorrhizal fungus of an achlorophyllous orchid, *R. gardneri* R. S. Rogers, which was provisionally classified as *Thanatephorus* (*T. gardneri* Warcup, sp. nov.) based on teleomorph morphology, formed ectomycorrhiza on *M. uncinata* R. Br., *Eucalyptus* spp., and *Poranthera microphylla* Brogn (Warcup, 1991). The study shows that the Ceratobasidiaceae fungus forms endomycorrhiza with the orchid *Cymbidium goeringii* and ectomycorrhiza with *Pinus Yunnanensis*, indicating these symbioses are not separate but host-dependent expressions of a single fungal species. The findings provide evidence of a tripartite symbiosis in which carbon may be transferred from pine to a photosynthetic orchid via shared mycorrhizal hyphae. The results obtained in this study would be in great importance regarding the conservation and propagation of *C. goeringii* in the natural habitats.

Declarations

Ethics approval and consent to participate

Seed collection of *Cymbidium goeringii* was approved by Administration Bureau of the Golden Temple National Forest Park Conservation and and the Gaoligong Mountains Conservation Station in Yunnan Province. Seed collection of *C. goeringii* was permitted by private land owners. The seedlings of the species were identifed by Lilan Deng, a taxonomist professorexpert at Southwest Forestry University. Voucher specimens of *C. goeringii* and *C. goeringii* have been deposited in the Southwest Forestry University with an accession number No. LXL-003 .This study complies with local and national regulations.The seedlings are tissue-cultured seedlings that we cultivated ourselves.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

DNA sequences□KMGT36□XXX, KMGT47□XXX, BSGLGM28:XXX

Credit authorship contribution statement.

Jianxing Chen: Methodology,Datacuration,Validation,Visualization,Writing-original

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Xiang Ma: Formal analysis,Visualization.

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Fang Wang: Writing-review&editing.

Xingliang Xu: Writing-review&editing.

Jianrong Wu: Conceptualization,Project administration, Funding acquisition,Writing-review&editing.

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Figures

Figure 1

Legend not included with this version.

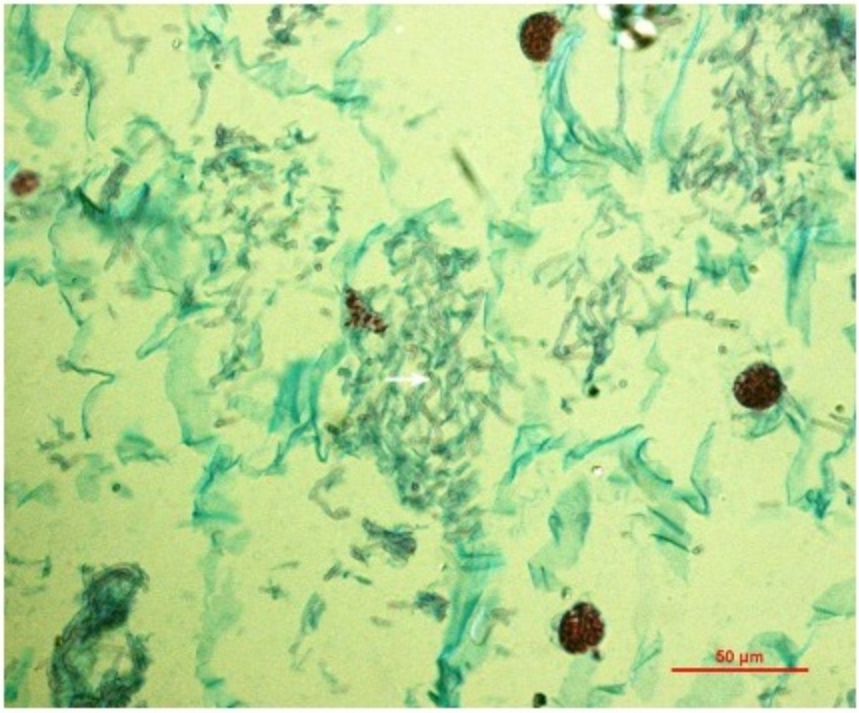

Figure 2

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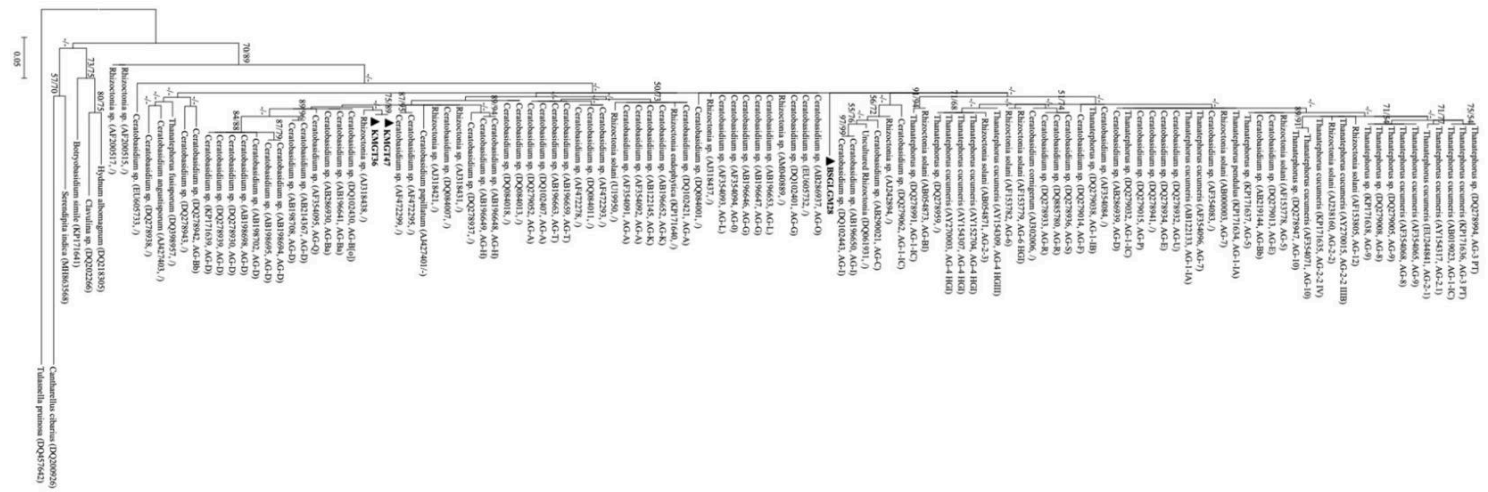

Figure 3

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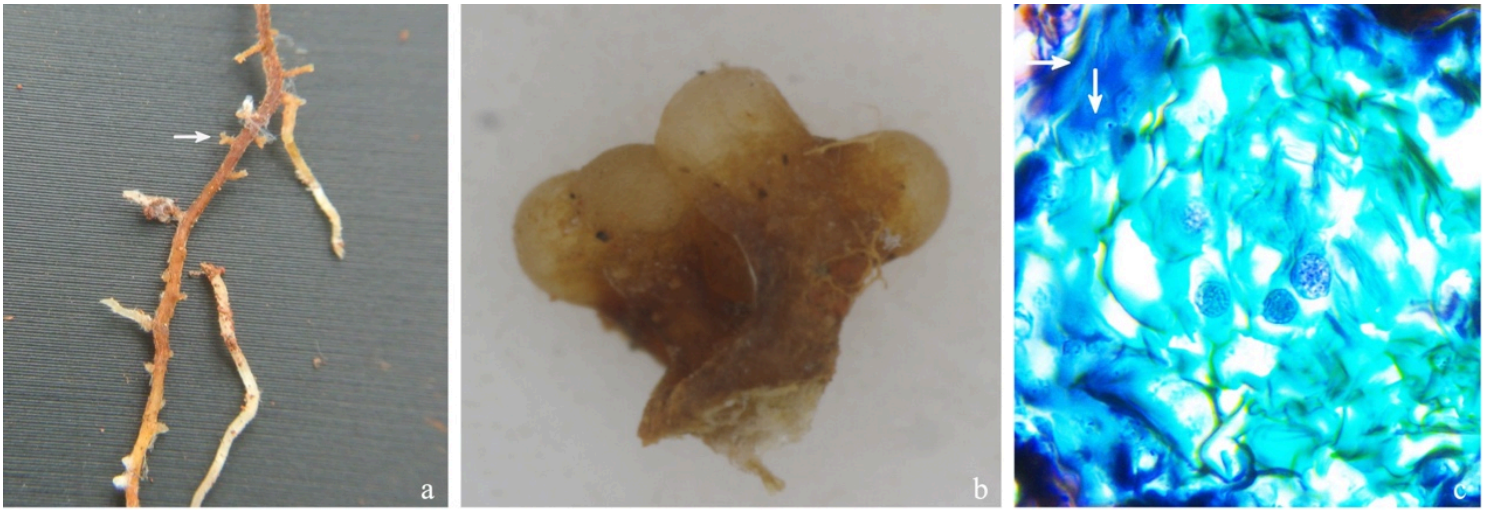

Figure 4

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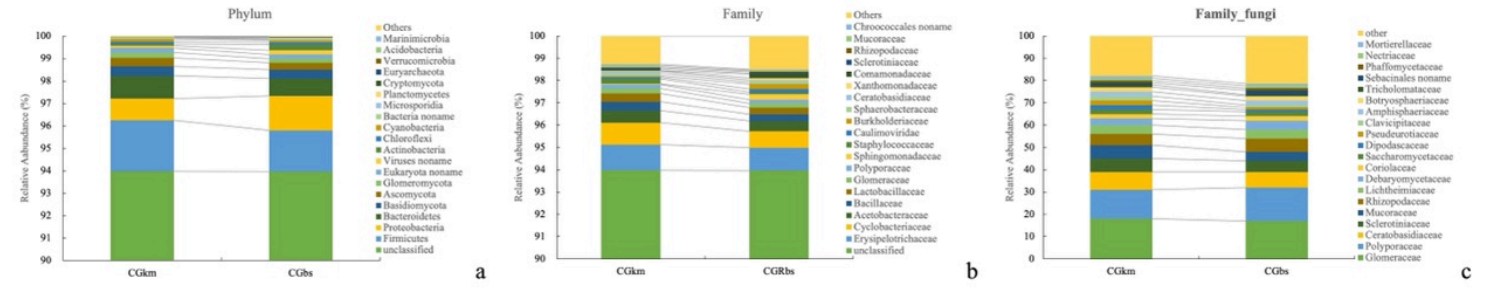

Figure 5

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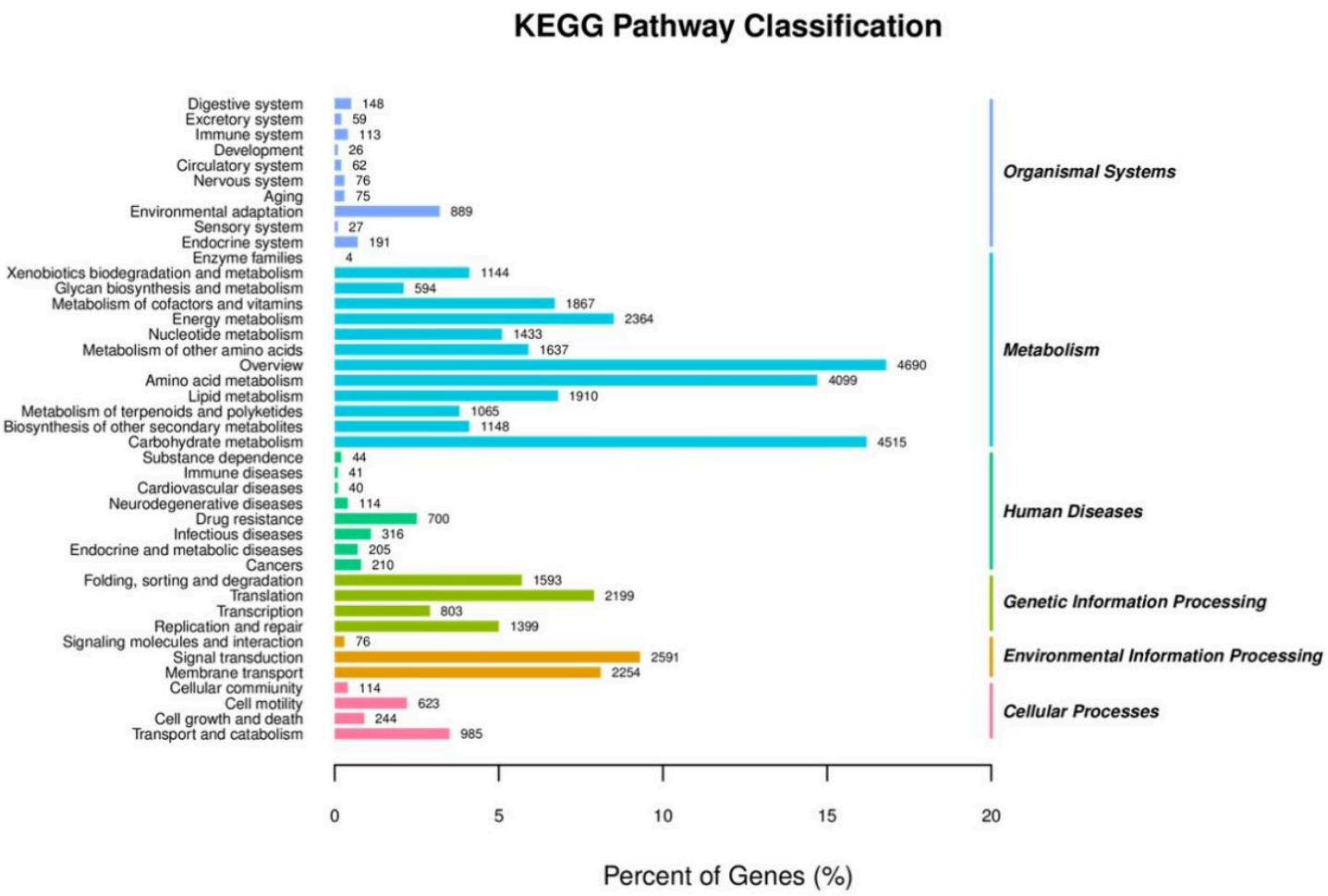

Figure 6

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