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研究报告

整合转录组数据鉴定大型真菌原基形成的潜在通路

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摘　要：【背景】大型真菌子实体发育特别是从菌丝体到原基转变的分子机制，目前仍不清楚。现有的研究大多集中在有限的真菌种类或环境因素上，但对在发育中起关键作用的基因提供的信息有限。【目的】研究大型真菌原基形成的分子机制。【方法】对 11 个物种及 4 个环境因子相关的转录组数据进行分析。【结果】与对照组相比，上调基因数量从白灵菇(*Pleurotus tuoliensis*)的 325 个到梅里克氏菌(*Rickenella mellea*)的 2 854 个，下调基因数量从白灵菇的 379 个到圆环蜜环菌(*Armillaria ostoyae*)的 3 189 个。根据 gene ontology (GO)注释，前 3 个生物过程类别为氧化-还原过程、代谢过程和碳水化合物代谢过程。此外，细胞成分类别中膜整体成分、核、膜等显著富集。同样地，分子功能类别包括水解酶活性、氧化还原酶活性和催化活性。【结论】大型真菌在原基形成中存在可能发挥重要作用的共同通路。

关键词：大型真菌；转录组测序；原基形成；环境因子

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Identification of potential pathways in primordium formation of mushroom-forming fungi: based on the analysis of RNA-Seq data

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Abstract: **[Background]** The molecular mechanism of fruiting body development in mushroom-forming fungi, especially the transition from mycelium to primordium, is still unclear. Most of related studies focused on limited fungal species or environmental factors, and offered little information on the key genes in the development of mushroom-forming fungi. **[Objective]** This paper aims to study the molecular mechanism for primordium formation of mushroom-forming fungi. **[Methods]** RNA-Seq data of 11 fungal species and 4 related environmental factors were analyzed. **[Results]** The number of up-regulated genes ranged from 325 (*Pleurotus tuoliensis*) to 2 854 (*Rickenella mellea*), while that of down-regulated genes was in the range of 379 (*Pleurotus tuoliensis*) to 3 189 (*Armillaria ostoyae*) compared with that of the control. As for the gene ontology (GO) terms, the top three biological processes were oxidation-reduction process, metabolic process and carbohydrate metabolic process. The mainly involved cellular components were the integral component of membrane, nucleus, and membrane, and the related molecular functions were hydrolase activity, oxidoreductase activity and catalytic activity. **[Conclusion]** There are some common key pathways for the primordium formation among mushroom-forming fungi.

Keywords: mushroom-forming fungi; RNA-Seq; primordium formation; environmental factors

The mushroom-forming fungi (*Agaricomycetes*), which originated 350 million years ago, comprise >21 000 species[1]. Fruiting bodies of mushroom-forming fungi have immense importance in agriculture, ecology, and medicine; they represent an important and sustainable food source with favourable medicinal properties (e.g., antitumor, immunomodulatory)[2]. The transition of mushroom-forming fungi from the mycelium to the primordia, which needs more energy than simple vegetative growth[3], is the most complex and critical developmental event. Understanding the mechanisms underlying primordia formation has long been a goal of research on edible mushrooms[4].

Recently, studies on *Pleurotus tuoliensis*[5], *Pleurotus eryngii*[6], *Lentinula edodes*[7], *Schizophyllum commune*[8], *Flammulina velutipes*[9], *Ganoderma lucidum*[10], *Cordyceps militaris*[11], *Hypsizygus marmoreus*[12-13], *Termitomyces heimii*[14], *Coprinopsis cinerea*[4,15], *Pleurotus ostreatus*[16], and *Sparassis latifolia*[17-19], found some key genes that regulated mushroom development and demonstrated the molecular mechanisms in this process, including hydrophobins[20-23], defense-related proteins[15], fungal cell wall (FCW) modifying enzymes[24-27], transcriptional regulators[8,28-29], and light receptors[11,18,30-31]. Since most of these studies focused on a single factor and/or single species,

they provide little information on what genes play a key role in the development of mushroom-forming fungi.

A new study[32] charted the transcriptomic landscape of multicellular development in six phylogenetically diverse mushroom-forming species and performed comparative analyses of >200 genomes, pinpointed nearly 300 conserved gene families, and another 73 gene groups with developmentally dynamic expression in five or more species, as well as 631 domains significantly overrepresented in mushroom-forming fungi, and investigated the general evolutionary and functional properties of fruiting body development using comparative transcriptomes of fruiting bodies of mushroom-forming fungi. However, the number of species was still limited, and they did not consider environmental factors.

So, in this study, we collected more RNA-Seq data related to the primordia formation. By integrative analysis, we found that genes involved in integral component of membrane, nucleus, and oxidoreductase activity were important for primordia formation. Such data should help define the conserved genetic programs underlying primordia formation in mushroom-forming fungi and provide new ideas for developing new cultivation mushroom species.

1 Materials and Methods

1.1 Data collection

Through a literature search, the accession numbers of RNA-Seq data related to the primordium differentiation of edible fungi and environmental factors were found out in the database of gene expression omnibus (GEO) and sequence read archive (SRA), and the original data were downloaded for further analysis. Most of these studies had three replicates in each group, but the studies of *Lentinula edodes*, *Hypsizygus marmoreus* and *Pleurotus eryngii* only had one sample in each group.

1.2 Protein-coding gene prediction of *Pleurotus tuoliensis*

Because of the lack of gene annotation information in *Pleurotus tuoliensis*, the gene models of the genome were predicted. The *Pleurotus tuoliensis* genome assembling data was downloaded from the joint genome institute (JGI) database (https://genome.jgi.doe.gov/portal/). Firstly, the homolog-based approach, Augustus V2.5.5[33], GeneMark-ES V3.0.1[34] and SNAP[35] software were utilized for gene prediction separately. Then, the results were integrated by software EVM[36] to predict all genes of the genome to obtain the final gene set.

1.3 RNA-Seq data analysis

Raw data (raw reads) of RNA-Seq were downloaded from the database. Raw data of FastQ format were filtered by Cutadapt[37]. Files were then processed by FastQC[38]. Reference genomes were directly downloaded from the NCBI genome website[39]. The reference genome index was built, and paired-end clean reads were aligned to the related genome using STAR[40]. The reads numbers mapped to each gene were counted using HTSeq V0.6.1[41]. Subsequently, we performed principal component analysis (PCA). The main principles are as follows: (1) The correlation within each group needs to be above 0.8, and the removal is less than 0.8. (2) When the correlation of each group is greater than 0.8 but less than 0.9, samples with a correlation difference of 0.5 are removed. (3) When the correlation between the experimental group and the control group is greater than 0.9, the samples with a correlation difference of 0.5 are removed. The DESeq2 R package was used to analyse the differential expression[42]. We used ‘FDR≤0.05 and $|\mathrm{Log}_2\ \mathrm{FC}|\geq 2$’ as the threshold to judge the significance of gene expression differences.

1.4 Function annotation

BLAST2GO (http://www.blast2go.com/b2ghome)[29] was used to obtain gene ontology (GO) annotations of unique assembled transcripts for defining biological processes, molecular functions and cellular components. The *P* value in

multiple tests was determined by the value for the false discovery rate (FDR). A GO term was considered a significant enrichment of differential proteins if the FDR was≤0.01.

2 Results and Analysis

2.1 Data summary

The RNA-Seq data of primordium differentiation of mushroom-forming fungi and related references were shown in Table 1 and Table 2. In total, 11 RNA-Seq datasets related to primordium differentiation from different species and 4 RNA-Seq datasets related to environmental factors were collected. The RNA-Seq data for *Flammulina velutipes* (SRP100485) could not be downloaded from the database, so we directly got the annotated DEGs data from professor Lin Junfang (South China Agricultural University, Guangzhou, China).

2.2 Result of protein-coding gene prediction for *Pleurotus tuoliensis*

In total, 14 615 protein-coding genes were predicted, characterized by an average gene length of 1 989.03 bp and an average exon number of 6.4. The genes predicted formed transcripts with an average length of 2.0 kb. The average exon, CDS and intron lengths were 230.9, 1 475.50 and 95.28 bp, respectively. However, the number of genes was 30 579 unigenes, with an average length of 1 347 bp in the previously reported[5]. These differences may because of the methods used.

2.3 Quality of RNA-Seq data

Hypsizygus marmoreus, *Lentinula edodes*, *Pleurotus eryngii* had only two samples and no correlation map; The expression matrix of *Ophiocordyceps sinensis* was downloaded directly from the database; List of DEGs of *Flammulina velutipes* was provided by professor Lin Junfang. Data for *Sparassis latifolia* was from our previous study[44]. The correlation results of the rest 8 RNA-Seq data were shown in Figure 1 and Figure 2. Depend on the criteria, GSM3564745 in *Coprinopsis cinerea*,

表 1 不同物种原基形成相关的转录组数据

Table 1 RNA-Seq data related to primordium differentiation from different species

物种 Species	菌丝 Mycelia	原基 Primordium	参考文献 References
Coprinopsis cinerea	GSM3564744	GSM3564750	[32]
	GSM3564745#	GSM3564751	
	GSM3564746	GSM3564752	
Lentinus tigrinus	GSM3564887#	GSM3564890	[32]
	GSM3564888	GSM3564891	
	GSM3564889	GSM3564892	
Rickenella mellea	GSM3564952#	GSM3564955	[32]
	GSM3564953	GSM3564956	
	GSM3564954	GSM3564957#	
Armillaria ostoyae	GSM2674732	GSM2674720	[32]
	GSM2674733	GSM2674721	
	GSM2674734	GSM2674722	
Phanerochaete chrysosporium	GSM3565037	GSM3565040	[32]
	GSM3565038	GSM3565041	
	GSM3565039#		
Schizophyllum commune	GSM3565023	GSM3565026	[32]
	GSM3565024	GSM3565027	
	GSM3565025	GSM3565028	
Pleurotus tuoliensis	SRR4293817	SRR5056086	[5]
	SRR4293819	SRR5056087#	
	SRR4293821	SRR5056088	
Lentinula edodes	SRR5891392	SRR5891393	[7]
Hypsizygus marmoreus	SRX685968	SRX685970	[12]
Ophiocordyceps sinensis	GSM3494866#	GSM3494872	[43]
	GSM3494867	GSM3494873	
	GSM3494868	GSM3494874	
Sparassis latifolia	GSM3728933	GSM3728939	[44]
	GSM3728934#	GSM3728940#	
	GSM3728935	GSM3728941	

注：#：基于主成分分析和皮尔森相关性分析这些数据被排除在后续的分析里面

Note: #: These data were excluded in further analysis depend on the principal component analysis (PCA) and Pearson correlation analysis.

表 2　环境因子相关的转录组数据

Table 2　RNA-Seq data related to environmental factors

物种 Species	转录组数据 RNA-Seq data		参考文献 References
	处理 Treatment	数据集 Data set	
Pleurotus eryngii	Dark	SRR3388145	[6]
	Blue light	SRR3388300	
Lentinula edodes	White	SRX4310575	[20]
		SRX4310576	
		SRX4310577	
	Brown	SRX4310578	
		SRX4310579	
		SRX4310580	
Flammulina velutipes		SRP100485	[45]
Sparassis latifolia	Light	GSM3728936#	[44]
		GSM3728937	
		GSM3728938	
	Dark	GSM3728933	
		GSM3728934#	
		GSM3728935	

注：#：基于主成分分析和皮尔森相关性分析这些数据被排除在后续的分析里面

Note: #: These data were excluded in further analysis depend on the principal component analysis (PCA) and Pearson correlation analysis.

GSM3564887 in *Lentinus tigrinus*, GSM3564952 in *Rickenella mellea*, GSM3565039 in *Phanerochaete chrysosporium*, SRR5056087 in *Pleurotus tuoliensis*, and GSM3494866 in *Ophiocordyceps sinensis* were excluded in the following differential expression analysis.

2.4　Analysis of differentially expressed genes (DEGs)

The results of differential expression analysis were shown in Table 3. The number of up-regulated genes was ranged from 325 in *Pleurotus tuoliensis* to 2 854 in *Rickenella mellea*, while the number of down-regulated genes was ranged from 379 in *Pleurotus tuoliensis* to 3 189 in *Armillaria ostoyae* compared with the control group.

2.5　GO annotation results

Based on the analysis of RNA-Seq data, the DEGs and GO annotations were firstly statistically sorted based on the results of the differential gene analysis; then, based on the corresponding GO annotation results, the statistics of each species or treatment in each GO term were calculated to find the GO term. Since there were too many Go term annotation results, the overall genes were sorted firstly, and then the top 30 annotation results were selected for plotting (Figure 3). The top 5 enriched GO categories were the integral component of membrane, nucleus, oxidoreductase activity, hydrolase activity, and carbohydrate metabolic process.

The top 50 GO terms extracted from the GO annotations of all the differential genes; the plot was shown in Figure 4. According to the GO annotation, the top three biological process categories were oxidation-reduction process, metabolic process, and carbohydrate metabolic process. In addition, cellular component categories were significantly enriched in the integral component of membrane, nucleus, membrane, et al. Similarly, the molecular function categories included hydrolase activity, oxidoreductase activity, and catalytic activity.

3　Discussion and Conclusion

Understanding the molecular mechanisms regulating the fruiting process in mushroom-forming fungi, especially the transition from mycelium to primordia form, has long been a goal in mycological research. The process not only requires the aggregation of hyphae to form three-dimensional structures and leads to the differentiation of several fruiting bodies-specific cell types not present in the vegetative mycelium[46], but also requires precise integration of a number of fundamental biological processes under special environmental conditions and is controlled by many developmentally regulated genes[47]. Many studies had focused on the mechanisms of fruiting body formation[5-7,9,12,14,16,23,32,44-45,48-57]. Since most of

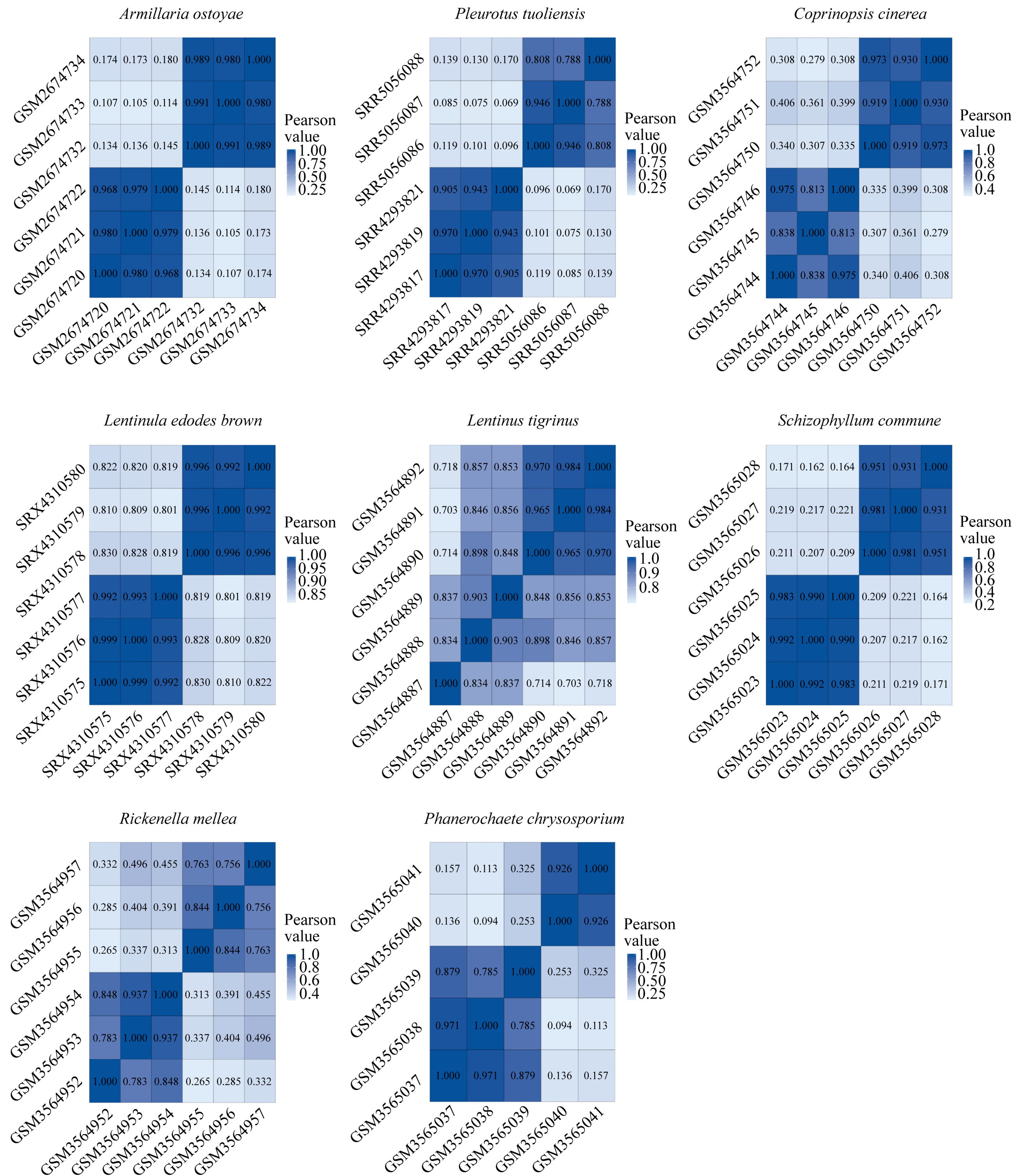

图 1　皮尔森相关性结果热图

Figure 1　The Pearson correlation results were shown by heatmap.

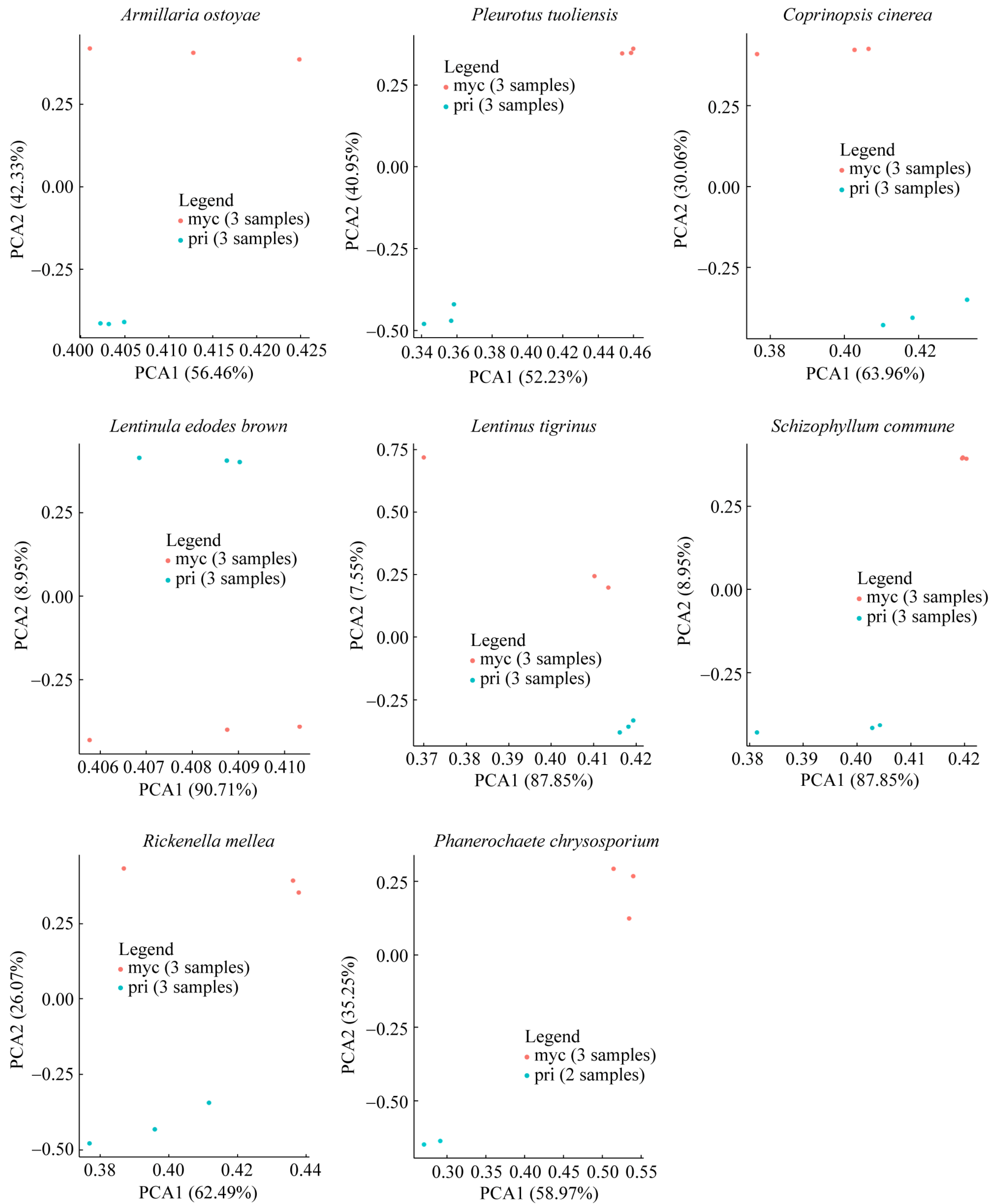

图 2 主成分分析图

Figure 2 PCA analysis plot.

表 3 差异分析结果

Table 3 Results of differential expression analysis

物种 Species	上调 Up-regulated	下调 Down-regulated
Armillaria ostoyae	2 760	3 189
Pleurotus tuoliensis	325	379
Coprinopsis cinerea	1 747	1 990
Hypsizygus marmoreus	938	1 684
Lentinula edodes brown	817	420
Lentinula edodes	1 197	598
Lentinus tigrinus	666	1 006
Ophiocordyceps sinensis	1 579	1 215
Phanerochaete chrysosporium	1 532	1 592
Pleurotus eryngii	2 125	670
Rickenella mellea	2 854	2 890
Schizophyllum commune	2 213	2 375
Flammulina velutipes	1 000	1 000
Sparassis latifolia PVSD	1 819	1 609
Sparassis latifolia LVSD	827	875

these studies focused on a single species, they provide little information on what genes play key roles in the development of mushroom-forming fungi. A new study[32] charted the transcriptomic landscape of multicellular development in six mushroom-forming species. However, the number of species was still limited. Therefore, we collected RNA-Seq data from more than 10 species of mushroom-forming fungi, which was almost double than that study.

Fruiting body development is triggered by changing environmental variables (e.g., nutrient availability, temperature, light), and involves a transition from vegetative mycelium to a complex multicellular fruiting body initial. Some studies also focused on the influence of light[5-6,20,44,58-59] and temperature[16,45,59]. However, there were very limited studies that combined the development stages and environment factors. In the present study, we not only considered the development stages and environmental factors but also combined the data from more than 10 species. Our results implied that genes involved in GO categories of integral component of membrane, nucleus, oxidoreductase activity, hydrolase activity, and carbohydrate metabolic process were important for fruiting body development. Hydrolases were found to be developmentally regulated in the previous study[32]. Hydrolase activity terms were also significantly enriched with down-regulated genes in the partial brown phenotype with light-induced mycelial browning in *Lentinula edodes*[20], which was an important process for ensuring the quantity and quality of this edible mushroom. This study also found that carbohydrate metabolic process term was significantly enriched with up-regulated genes in the brown phenotypes. In *Agaricus blazei*, GO terms of integral component of membrane, oxidoreductase activity, carbohydrate metabolic process, and hydrolase activity were found out by comparing the GO annotation of DGEs between mycelia and primordia[57], which was consistent with our results. What's more, GO terms related to oxidoreductase activity, and carbohydrate metabolism were shared by six species during development[32].

However, there were still some limitations in our study. First of all, the number of data collected was limited. There were some newly published data not included. For instance, the data for *Agaricus blazei*[57] and *Ophiocordyceps sinensis*[48]. Second, the quality of sequencing was different. The method and depth of sequencing were not all the same, and the genome annotation level of different species was different. Third, the data analyzed in this study were all RNA-Seq data, not including the other omics data. For example, proteomic data in *Flammulina velutipes*[9], *Cordyceps sinensis*[54] and *Termitomyces heimii*[14], and ATAC-Seq (assay for transposase accessible chromatin by sequencing) data in *Sparassis latifolia*[44]. We should be able to achieve more valuable results if we can overcome these shortcomings.

Acknowledgments: We want to thank professor Lin Junfang (South China Agricultural University, Guangzhou, China) for his providing the annotated DEGs data of *Flammulina velutipes*.

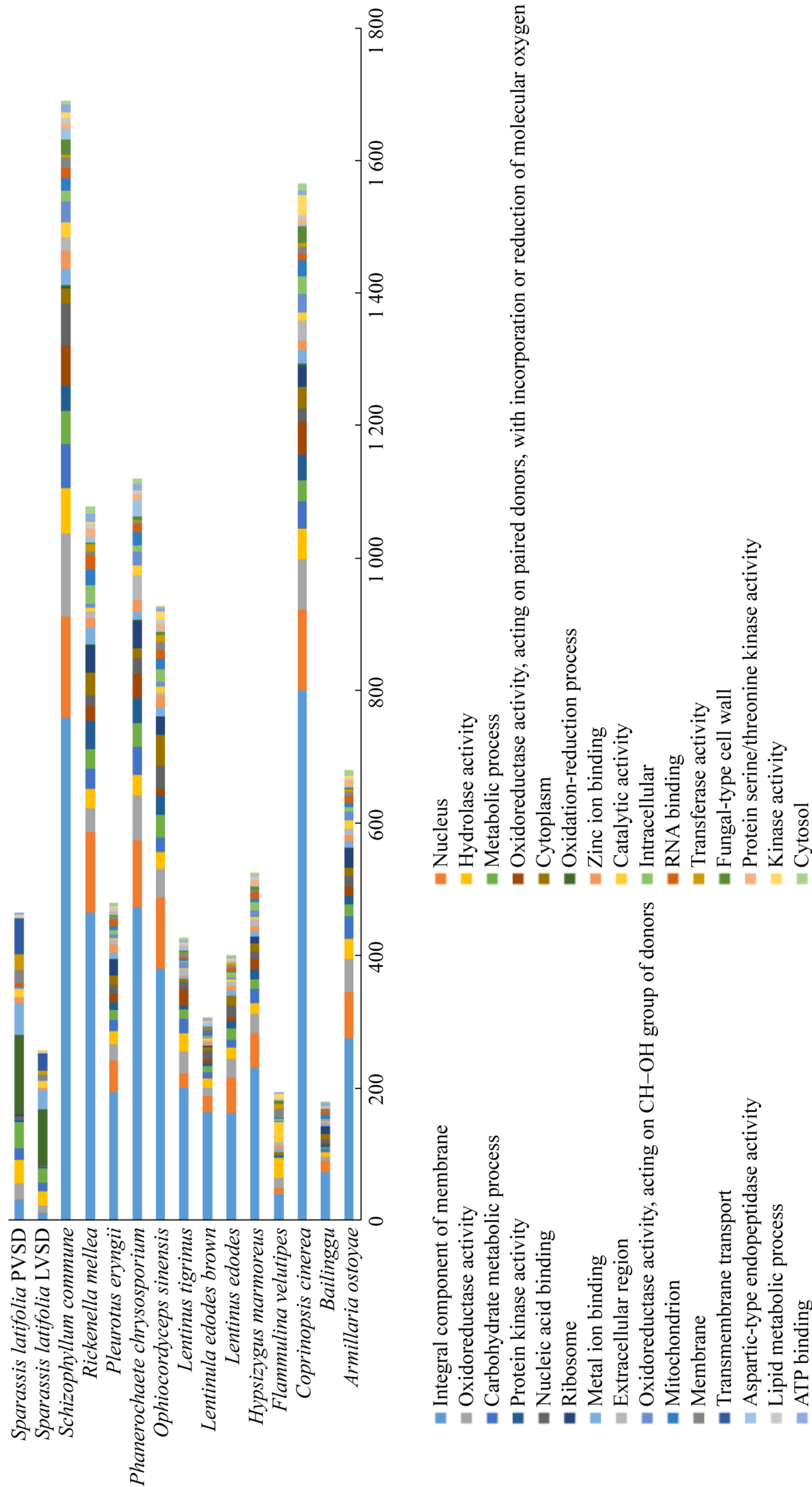

图 3 来自 15 个转录组数据集的差异基因 GO 条目的统计图

Figure 3 GO term statistics of DEGs from all 15 RNA-Seq dataset.

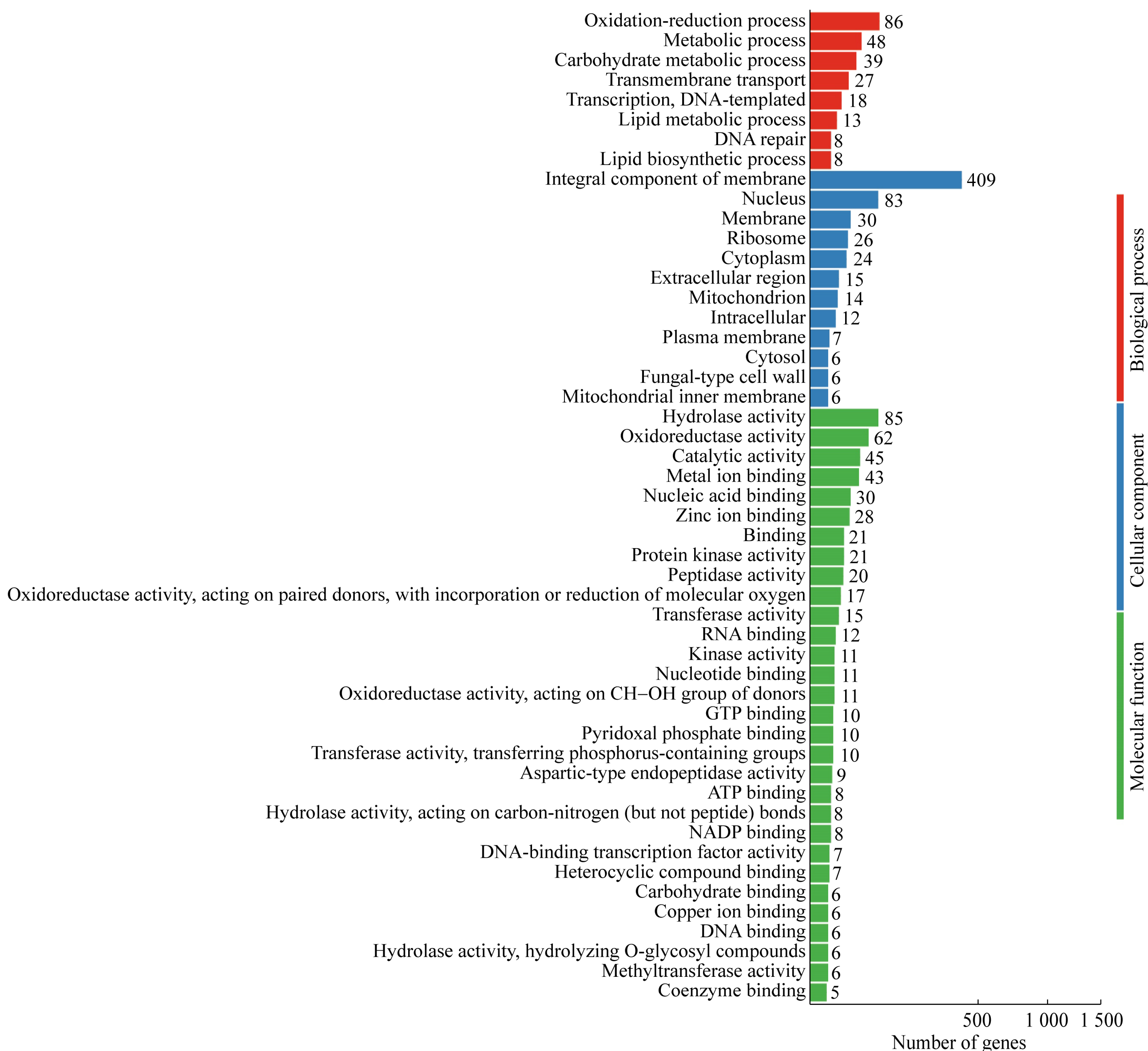

图 4　所有差异基因的 GO 分布图

Figure 4　GO statistics chart of all differential genes.

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