Phylogenetic Analysis and Cultivation Technique of *Ganoderma lucidum* from China

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Abstract: *Ganoderma* strains are wood-rotting fungi and popular living in tropical forest. Based on the morphological characteristics, phylogenetics analysis of rDNA-ITS and mitochondrial small-subunit ribosomal DNA sequences, we proposed isolates collected from *Cyclobalanopsis bambusaefolia* grown in China were *G. lucidum*, also this group isolates could be artificial cultivation. Morphology, host distribution and molecular phylogenetic evidence supported recognition of this species. Phylogenetic analysis indicated these isolates clustered with Asian *G. lucidum* into a distinct clade. Artificial cultivation showed fruiting bodies were thick, corky, and yellowish in the growing margins and then turn to brownish in the matured part on the surface.

Key words: phylogenetics; rDNA-ITS; mt SSU; *Ganoderma lucidum*
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Ganoderma was a saprophytic and thoroughly studied genus among pore fungi. They could decay lignin and cellulose, result in worldwide losses of crops and trees[1-2]. Recent application of modern analytical techniques has indicated *Ganoderma* was composed of a vast number of bioactive compounds, such as triterpenes, polysaccharides, bioactive proteins and other substances.

Phylogenetic studies the sequences of rDNA-ITS and mitochondrial small-subunit ribosomal DNA (mt SSU) proved that *Ganoderma* species were divided into six monophyletic groups, most members of common species clustered in the same clade[3]. In other studies, a new species *G. carocalcareus* was proposed, and this species distinct in *G. resinaceum* group, but had close relative of *G. subamboinense* and *G. weberianum*[4]. These results indicated that rDNA-ITS and mt SSU sequences were useful for the phylogenetic analysis of *Ganoderma* genus[5].

Wild *Ganoderma* fungi could be divided into 103 species, 72 kinds were found in Hainan, China. Tropical and subtropical rain forest of Hainan provided a suitable temperature, humidity and adequate nutrient[6]. However, there were little phylogenetic reports of *Ganoderma* isolates, especially, artificial cultivation of *Ganoderma* fungi from Hainan[7-9]. We explored the phylogenetic relationships of 2 *Ganoderma* isolates based on rDNA-ITS and mt SSU sequences, and developed artificial cultivation technique of this strain.

1　MATERIALS AND METHODS

1.1　Test material

Ganoderma fungi were collected from Jianfengling Hainan, China.

1.2　Test method

1.2.1　Disinfection treatment　Isolates obtained from *Cyclobalanopsis bambusaefolia* together with representative isolates were selected for phylogenetic analysis. Segments of fruiting bodies were disinfected by double treatments of 75% alcohol and NaOCl (1% available Cl). After rinsing in sterile water, the samples were placed on potato-dextrose agar (PDA) and incubated at 25℃ in dark. After 1～2 weeks incubation, colonies developed from the cut injuries of samples. Fungal isolates were stored at 4℃.

1.2.2　Morphology and cultivation technique　Isolates were cultured at 25℃ on PDA; morphological characters of fungi were measured after 2 weeks incubation[10]. The process for producing fruiting bodies was divided into two major stages. During first stage, isolates was cultivated on three different substrates respectively at 25℃, ingredient was as follow: ①PDA, 1 week of cultivation; ②73% saw-

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dust, 20% wheat bran, 5% soybean powder, 1% cane sugar, and 1% gypsum, 3 weeks of cultivation; ③ Short logs about 15 cm long and 10 cm in diameter, 7 weeks of cultivation. Second stage, the prepared logs were buried in soil; the soil allowed optimum conditions of drainage, air permeability, and water retention. The moisture content of the greenhouse was about 60% to 90%, and temperature was between 25℃ and 35℃. After 9 weeks, the fruiting bodies were appeared and achieved mature status. Parameters of characteristics were measured, such as fresh weight and dry weight, etc.

1.2.3 DNA extraction, amplification and sequencing Genomic DNA was extracted by potassium acetate and stored at 4℃[10]. Amplification of rDNA-ITS fragments was carried out with a MyGene™ Series Peltier thermal cycler (LongGene, Hangzhou, PRC). ITS1, 5.8S and ITS2 segments were amplified with primers rDNA-ITS1 and rDNA-ITS4[11]. Partly mt SSU sequences were amplified with primers CFMT (5′-AAGGTGTAGAGGTGAGTATG-3′) and CRMT (5′-TTCGTTTCTCAGCGTCAATC-3′). And mt SSU included complete variable domain V3, V4 and portion V2 (Hong & Jung 2004). PCR amplification, product separation and purification were carried out as described by Li[10]. DNA purification was sequenced by Beijing Genomics Institute (Beijing, PRC).

1.3 Data analysis

DNA sequences were analyzed by DNAssist 2.2[12]; Clustal X 1.81[13] then manually adjusted if necessary. Alignment gaps and ambiguous characters were treated as missing information. Maximum parsimony (MP) analyses were performed in MEGA 4[14], in which all characters were unordered and reversible. We conducted close neighbor interchange search methods for finding the optimal trees under the MP criterion. To assess the robustness of the topology 1 000 bootstrap replicates were run by MEGA 4.

2 RESULTS AND ANALYSIS

2.1 Characteristics of cultivated fruiting bodies and colonies

Colonies cultured on PDA were white, cottony, and circular with a regular margin, bright yellow reverse and smaller proportion of fibrous hyphae. Ninety-three sterilization logs of Jfl1 were buried in soil, and thirteen logs produced fruiting bodies. Likewise, 29 logs of Jfl3 produced the fruiting bodies among 70 logs. Cultivars generally experienced four stages: primordia formation, stipe formation, cap formation, fruiting body maturity (Fig 1 A, B, C, D). Fruiting bodies were thick, corky, and the margins were yellowish, and then turn to brownish in the matured part on the surface. Characteristics of matured Jfl1 and Jfl3 fruiting bodies were listed in Table 1.

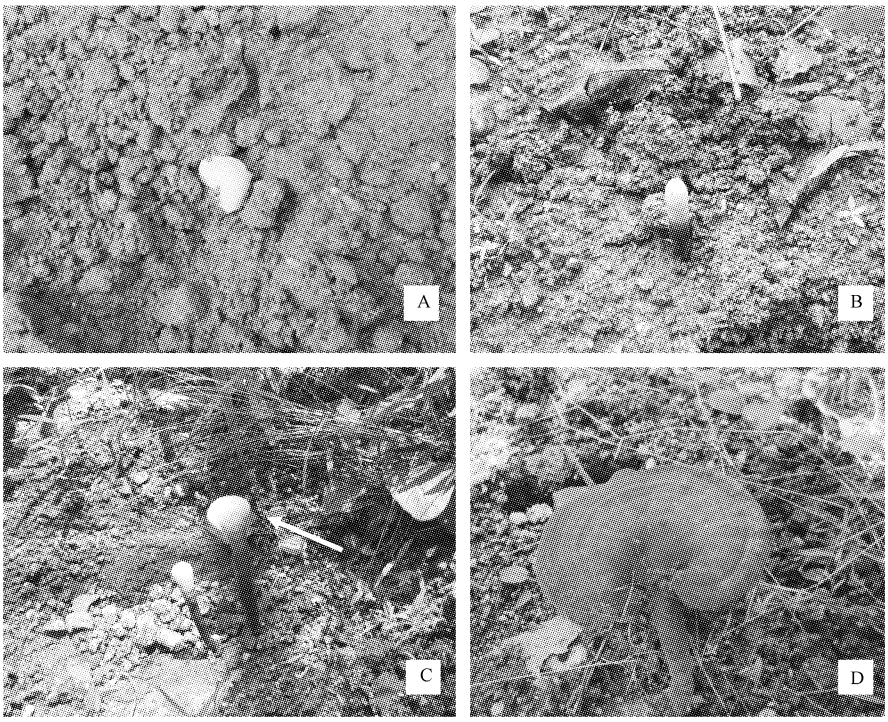

Fig. 1 Characteristics of Jfl3 fruiting body grow in experience stage

Note: A. Primordia formation; B. Stipe formation; C. Pileus formation, arrow represents pileus; D. Matured fruiting body.

Table 1　Characteristics of fruiting bodies of matured *Ganoderma lucidum*

Isolates	Stipe length/cm	Stipe width		Pileus radius/cm	Pileus thickness/cm	Wet weight/g	Dry weight/g
		Top/cm	Root/cm				
Jfl1	6.0	1.1	1.0	5.3	0.7	16.8	12.1
Jfl3	6.8	1.0	0.8	4.1	0.5	9.4	7.9

2.2　Phylogenetic relationships

PCR amplification of rDNA-ITS and mt SSU from *Ganoderma* isolates genomic DNA yielded products of 542 bp and 515 bp respectively. The products of rDNA-ITS included complete internal transcribed spacer (ITS1-5.8S-ITS2) and portion of 18S,25S. DNA sequences of mt SSU fragments in Jfl1,Jfl3 were identical. DNA sequences were accepted in GenBank and the accession numbers are HQ689695,HQ689696,JX966190,and JX966191.

Phylogenetic analyses were conducted to determine the relationships of Jfl1 and Jfl3 with other *Ganoderma* species. Phylogenetic tree of rDNA-ITS revealed two *Ganoderma* isolates Jfl1 and Jfl3 clustered with *G. lucidum* WD565 and *G. lucidum* NBRC31863 in one distinct clade,and supported in 93% of the bootstrap replicates. Most members of a particular *Ganoderma* species tended to cluster together. For example,the isolates of *G. fornicatum*,*G. pseudoferreum*,*G. tenue*,*G. cupreum*,*G. gibbosum* and *G. applanatum* grouped into exclusive clades with high bootstrap value (Fig. 2). Phylogenetic tree based on mt SSU was little different with rDNA-ITS. Isolates Jfl1,Jfl3 clustered with *G. lucidum* KCTC6279 with weaker bootstrap value of 70%. And this clade was also significantly different from other *Ganoderma* species (Fig. 2).

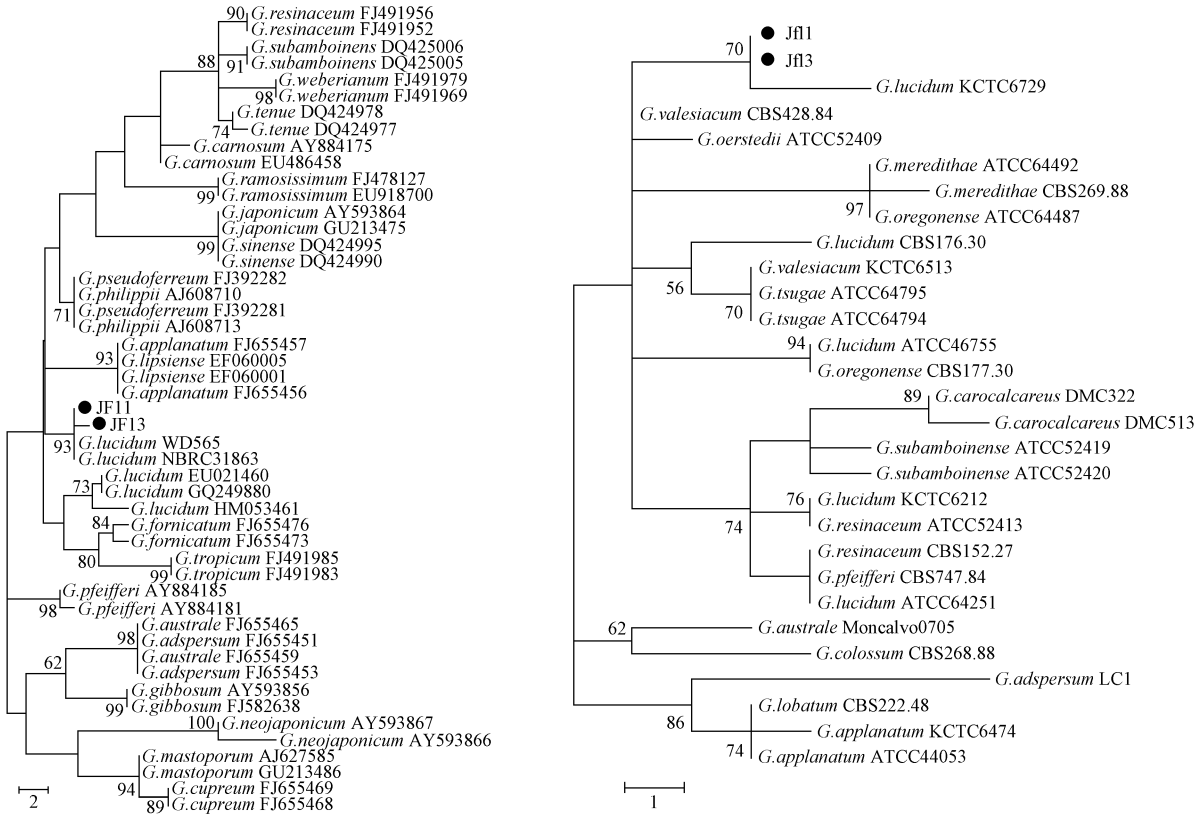

Fig. 2　*Ganoderma* spp. rDNA-ITS and mt SSU phylograms based on maximum parsimony (MP)

Note:Numbers (>50%) at branches are the percentage of trees containing the corresponding clade based on 1 000 bootstrap replications. rDNA-ITS MP tree shown:tree length=109 steps,consistency index=0.653,retention index=0.894,composite index=0.607. mt SSU MP tree shown:tree length=43 steps,consistency index=0.828,retention index=0.921,composite index=0.814. Circles represent isolates grown in *C. bambusae folia*.

3　DISCUSSION

Most members of *Ganoderma* species tended to cluster together in the rDNA-ITS and mt SSU phylogram. Phylogenetic analyses revealed isolates Jfl1 and Jfl3 was grouped with Asian *G. lucidum*, and exclusively from other species, indicating these 2 isolates had a close

relationship with Asian *G. lucidum* and had the genetic dissimilarities with other species (Fig. 2). Compared to the isolate *G. lucidum* KCTC6729 obtained from *Quercus gilva* rotting stump in Japan, Jfl1 and Jfl3 showed similar characteristics farinaceous texture, smaller proportion of fibrous hyphae and compact pseudoparenchyma of abundant cuticular cells[3]. In addition, typical morphological features of *G. lucidum*, thick, corky, and yellowish, were observed from the fungal isolates Jfl1 and Jfl3. Based on phylogenetic evidence and fungal morphology, we proposed the isolates found in *C. bambusae folia* native to China as Asian *G. lucidum*.

The technique to cultivate *Ganoderma* fungi was successfully achieved in the early 1970s, and production had developed rapidly in China since 1980[15-18]. However, most members of *Ganoderma* fungi to be cultivated successfully were divided into *G. lucidum*. The reports about other species were rare, especially about the isolates native Hainan could be cultivated[19-20]. In this research, we reported a group of isolates grown in Hainan could be cultivated, though only 13 and 29 sterilization logs of Jfl1 and Jfl3 could produce fruiting bodies respectively. Previous studies revealed *Ganoderma* fungi were sensitive to carbon dioxide. The air carbon dioxide level were raising 0.1% to 1%, branching of stipes was stimulated, but pileus formation was inhibited, and pileus formation re-activated after carbon dioxide removed[19]. In the research, we also found the similar characteristics. And cultivated pileus of Jfl1 and Jfl3 showed yellow and brown, were different with the wild which were red, pink (Unpublished). It indicated artificial cultivation turn the feature of pileus. The technique could be helpful for other species's cultivation[4,7,21]. And further study still need to increase yield of fruiting bodies and improve characteristics.

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激素配比对草莓叶片不定芽分化的影响

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摘　要：以“晶瑶”草莓叶片为外植体，研究了不同激素配比、$AgNO_3$ 浓度和暗培养对草莓叶片不定芽分化的影响。结果表明：当培养基为 MS＋噻二唑苯基脲(TDZ) 1.0 mg/L＋吲哚丁酸(IBA) 0.4 mg/L＋$AgNO_3$ 2.0 mg/L 时，不定芽再生率高达 70.3%，每个外植体平均再生芽数为 4.2 个；$AgNO_3$ 可以有效抑制“晶瑶”草莓叶片玻璃化和褐变；暗培养处理促进“晶瑶”草莓叶片的不定芽诱导，最佳暗培养时间为 14 d。

关键词：草莓叶片；噻二唑苯基脲(TDZ)；暗处理；不定芽

中图分类号：S 668.4　　**文献标识码**：A　　**文章编号**：1001－0009(2014)08－0096－04

草莓(*Fragaria ananassa* Duch)属蔷薇科草莓属多年生草本植物，又叫洋梅，原产于欧洲，20 世纪初引进我国。草莓的生育周期短并且产量高，具有较高的经济价值[1]。以往草莓品种改良常采用常规的杂交育种技术，但这种育种方式周期长，选择效率低，再加上草莓有无性繁殖的特点，因此适合用基因工程技术进行育种。噻二唑苯基脲(N-phenyl-N-1,2,3-thiadiazol-5ylurea，TDZ)是德国 Schering 公司 1976 年合成的高效激素棉花脱叶剂[2]。TDZ 是一种高效的植物生长调节剂，在植物组织培养快速繁殖体系中应用广泛，主要用于离体再生，被认为是许多植物种类诱导植株再生的唯一生长调节物质[3-5]。近年来，诸多难以离体分化的植物因采用 TDZ 而达到高频分化。对于草莓叶片离体再生，除了注意品种的选择外，TDZ 当为首选激素，大量研究表明，TDZ 可促进草莓离体高频再生[6]。现以“晶瑶”草莓为试材，研究了不同激素配比、$AgNO_3$ 浓度和暗培养对草莓叶片不定芽分化的影响，以期为草莓的遗传转化提供理论依据和实践经验。

1　材料与方法

1.1　试验材料

供试材料为植物资源科学与绿色生产吉林省重点实验室繁殖的草莓(*Fragaria ananassa* Duch.)‘晶瑶’组培苗。所用试剂 TDZ 为 Sigma 进口分装试剂。

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中国产 *Ganoderma lucidum* 的系统发育学分析及栽培技术研究

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摘　要：灵芝是一类木腐真菌，广泛存在于热带雨林中。根据形态特征、rDNA-ITS 和 mt SSU的系统发育学分析，课题组将从 *Cyclobalanopsis bambusaefolia* 分离出来的菌株命名为 *G. lucidum*，该菌株可人工培养。系统发育学分析表明，这些菌株类群与亚洲灵芝为一个独立的分支；此外，该研究采用传统椴木栽培技术，实现了分离菌株的人工栽培。人工栽培灵芝子实体粗厚，木栓质，边缘淡黄，在成熟的过程中逐渐变褐。

关键词：系统发育学；rDNA-ITS；mt SSU；*Ganoderma lucidum*