Science and Cultivation of Edible Fungi, Van Griensven (ed.)
© 2000 Balkema, Rotterdam, ISBN 90 5809 143 0

Diversity of rDNA sequences indicates that China harbours the greatest germplasm resource of the cultivated mushroom *Lentinula edodes*

S.W.Chiu – *Department of Biology and Environmental Science Program, The Chinese University of Hong Kong, People's Republic of China*

W.T.Chiu – *Department of Psychology in Education, University of Pittsburgh, Pa., USA*

F.C.Lin – *Laboratory of Applied Mycology, Huazhong Agricultural University, Wuhan, People's Republic of China*

D.Moore – *School of Biological Sciences, The University of Manchester, UK*

ABSTRACT: Ribosomal DNA polymorphism has been used to type strains as well as for phylogeny analyses. In *Lentinula edodes*, the ITS regions are sufficiently conserved (Hibbett et al. 1995) to be used to trace lineage relationships. Hibbett et al. (1995 & 1998) identified five lineages in Asia-Australasia with two lineages (named group I and V) appearing in China. As the sample size of all these earlier studies was rather small, we carried out a large scale and more detailed collection program in China. This report will focus attention on three provinces that are not among those in which traditional cultivation is popular. These are central Hubei, north central Shaanxi and the most south west Yunnan. Several fruit bodies from a single fallen log and fruit bodies from different logs were collected and used for culture isolation (Chiu et al. 1999). Direct sequencing of the ITS regions as marked by fungal-specific primer set (ITS4 and ITS5) was performed. The results reveal that a dominant group I rDNA lineage appears in these three provinces. Isolates of the group V lineage previously reported in Nepal and Hubei were found in Shaanxi province instead. However, novel rDNA sequences (not belonging to any of the five lineages previously described) were found in the three provinces. Thus, several previously unsuspected rDNA lineages are present in China. Also, as isolates belonging to different rDNA lineages were found growing in one single log, it is evident that genetic recombination has freely occurred. This accounts for the high genetic polymorphism existing in the remote mountainous areas of China. Protecting the environment from unsympathetic exploitation is the best strategy for conserving this natural resource.

1 INTRODUCTION

The black oak mushroom *Lentinula edodes* (commonly called shiang-gu, shiitake) is native to China but its popularity, not just amongst Chinese or Japanese communities, is now worldwide. It is second only to *Agaricus bisporus* in world production. China is the major producer; the harvest in 1997 was recorded as 91,500 metric tons (dried) and accounted for about 70% of world production, with about one-third of the Chinese crop being exported (van Nieuwenhuijzen 1998; Wang 1998). *L. edodes* is valued for a unique flavor which derives mainly from its content of the modified amino acid lenthionine and the nucleotide guanine-5-monophosphate (Yang *et al.* 1998). The fruit bodies are also rich in minerals, essential amino acids (especially lysine and leucine), are high in fiber content but contain less than 10% crude fat (Ho, Hun & Yei 1994). Besides being a nutritionally valuable crop, *L. edodes* has pharmaceutical values; a protein-bound polysaccharide (lentinan) has been extracted from its fruit bodies and found to have clinically-useful immunomodulatory, anti-cancer and anti-viral effects (Chihara 1993; Anon. 1998; Mizuno 1999).

Lentinula edodes is a species only found in the Asia-Australasia region. A related species, *L. boryana* is found in Mexico and S. America (Shimomura *et al.* 1992; Fukuda et al. 1994; Hibbett et al. 1995, 1998). Cultivated strains of *L. edodes* collected worldwide were genetically heterogeneous (Kwan et al. 1992; Chiu, Kwan & Cheng 1993; Fox et al. 1994) in contrast to

the low variability in Chinese cultivars (Chiu et al. 1996). Hibbett et al. (1995, 1998) proposed that five rDNA lineages exist in the *L. edodes* population. Group I includes populations from northeast Asia to the south Pacific. Group II includes populations from Papua New Guinea, Australia and Tasmania. Group III is limited to New Zealand. Group IV is from Papua New Guinea. Group V is from central Hubei province (not eastern as suggested by the authors), China and Nepal. Their study, however, examined only seven Chinese strains.

Biodiversity in nature is a key element of the genetic resource for breeding programs and, consequently, hunting for wild *L. edodes* has been practiced for a long time (Mori, Fukai & Zennyoji 1974; Zhang & Lai 1993). However, discussion of the biodiversity and population structure of this economically important mushroom seems to be limited to our recent publication (Chiu et al. 1999). As cultivation of this mushroom is widespread in China, and there are over 10 million mushroom farmers across the country (Luo 1998), escape of commercial cultivars into the wild may pose a threat to the native germplasm (Hibbett et al. 1995). Thus a baseline survey of the genetic diversity is necessary. This assessment can provide an indication of whether special conservation efforts are needed for *L. edodes*. In addition, the collected wild germplasm is a resource that can be exploited for commercial use (Chiu et al. 1998).

2 MATERIALS AND METHODS

2.1 *Isolation of specimens*

Fruit bodies of *Lentinula edodes* (Berk.) Pegler were surveyed and collected in remote mountains or reserve areas in the provinces of central Hubei, north central Shaanxi and the most southwest Yunnan (Figure 1). The collection sites were at least 100 km away from the nearest residences). During collections in Hubei and Yunnan, only one fruit body was collected from a fallen tree trunk at a site, and collection sites were at least 5 km apart. Population structure on a much fine scale was studied in Shaanxi province, and all fruit bodies borne on two nearby fallen tree trunks were collected. The identifiable tree logs in the collection sites were: *Cyclobalanopsis glauca*, *Quercus variabilis*, *Liquidambar formosana*, *Castanea mollissima* and *Castanea sequinii*. The purified tissue culture from each fruit body was regarded as an isolate (Chiu et al. 1999)

2.2 *DNA extraction and Direct Sequencing*

DNAs were mini-prepared from mycelia (White et al. 1990; Yoon, Glawe & Shaw 1991; Yoon & Glawe 1993). Primers ITS 4 and 5 were used to amplify the portion comprising ITS1, 5.8S and ITS2 regions of the nuclear ribosomal DNA. Then, primers ITS 5 and ITS3 were used as sequencing primers to amplify ITS1 and ITS2 respectively (White et al. 1990). The specific PCR programme comprised: 95°C for 1 min; 60°C for 1 min and 70°C for 1 min for 39 cycles with the last extension time lengthened to 10 min. Then the dsPCR product was cleaned of

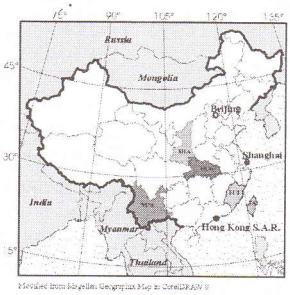

Figure 1. Collection of *Lentinula edodes* made in China. HUB, Hubei province; SHX, Shaanxi province; YUN, Yunnan province.

dNTPs and excess primers by glassmilk adsorption (Geneclean kit II, BIO 101). Purified PCR product as template was mixed with ABI PRISM^{TM} dRhodamine terminator cycle sequencing ready reaction kit containing AmpliTaq DNA polymerase, FS (Perkin Elmer) according to the following proportions: Terminator Ready Reaction Mix, 8 µl; PCR product, 20 ng; primer, 3.2 pmoles; and water added to a final volume of 20 µl. The thermal cycling programme for the sequencing reaction was: 25 cycles of 96°C for 10s, 50°C for 5 s and 60°C for 4 min. The sequence was read by an automated DNA sequencer (model 310; Perkin Elmer).

2.3 *Phylogenetic Analysis*

Analysis and alignment of the sequences were initially done using the clustral alignment command of the Perkin Elmer program Sequence Netvigator and fine-tuned manually. Reference sequences of the five rDNA lineage groups (I to V, according to Hibbett *et al.* 1995, 1998) downloaded from Genbank were also included in the analysis. Phylogenetic trees were obtained from the data by both distance and parsimony methods. For distance analysis, DNADIST in PHYLIP ver. 3.5 (Felsenstein 1989) was used to obtain a matrix of Kimura's two parameter distances (Kimura 1980). The strength of the internal branches from the resulting trees was statistically tested by bootstrap analysis from 100 bootstrap replications using the maximum likelihood program DNAML of PHYLIP. Consensus trees using the majority rule were constructed (Felsestein 1993; http://evolution.genetics.washington.edu).

3 RESULTS AND DISCUSSION

3.1 *Group I and V Haplotypes in China*

The predominant haplotype for the ITS1 and ITS2 sequences were the group 1 lineage designated by Hibbett et al. (1995, 1998). Thus, this group I rDNA lineage has now been found distributed in continental Chinese provinces from west to east: Zhejiang, Yunnan, Sichuan, Jiangsu, Shaanxi, Hubei, Anhui, and Fujian; in northern countries of China: North Korea and Japan, and to the south of China in Thailand and Borneo (this study together with Hibbett et al. 1995, 1998). Clearly, this lineage occupies the biggest territory and has China, the country with the longest history of mushroom cultivation in the center of its range.

A haplotype similar to the rDNA lineage group V (Hibbett et al. 1998) was found from three isolates from Shaanxi province, China. No sequence fell into the same clade as rDNA lineages designated as groups 2, 3 and 4. These latter lineages were only found in Papua New Guinea, Australia and Tasmania, and New Zealand (the Australasian region). Thus, geographical isolation seems to be leading to incomplete speciation through the appearance of ecotypes. The isolates from these regions are still compatible with those from Asian continent (Mori et al. 1974). Yet their sequences of nuclear rDNA and β-tubulin, and RFLPs of mitochondrial DNA were distinctive from those of Asian *L. edodes* strains (Hibbett *et al.* 1995, 1998; Fukuda *et al.* 1994; Thorn & Royse 1999).

3.2 *Novel rDNA Haplotypes in Chinese strains*

New and distinctive haplotypes were found in Hubei, Shaanxi and Yunnan (Figure 1). ITS1 and ITS2 of a strain evolves independently; e.g. a Hubei strain possesses ITS1 of group 1 type but a novel ITS2 haplotype. In addition, a few strains formed novel clades in the consensus trees, revealing novel lineages in provinces Shaanxi and Yunnan. Neither of these provinces is a traditional area for cultivation of shiang-gu and all collection areas were remote (a two day mountain trek from the nearest road). High mountains and great lake areas feature in these areas of continental China. The novel lineages we have detected suggest that genetic drift resulting from geographic isolation is leading towards incomplete speciation. Evidently the extensive and diverse remote areas of China harbour the greatest diversity of *L. edodes* and represent a great resource of heterogeneous germplasm of this economically important cultivated mushroom.

3.3 *Isolates of different lineages can be in close proximity*

In Shaanxi province, there were three clades, including the group 5 lineage, amongst isolates from the two trunks. In two cases, identical ITS1 and ITS2 sequences were shared among isolates from the same log. In another piece of the same trunk, variation was observed between the three isolates that were collected. These observations show that genetic recombination occurs without hindrance in areas like this that contain more than one lineages. It is to be expected that more and more novel lineages will be generated in time even though the sequences of the ITS1 and ITS2 regions are found to be stable in *L. edodes* (Hibbett et al. 1995, 1998; Nicholson et al. 1997).

Genetic diversity is created by mutation and filtered over history by biological, demographic and historical processes (Chakravarti 1999). The existing sequence variation is non-random but is shaped both by chance and natural selection, and by the demographic organization and migrations and dispersal patterns. China is geographically diverse; she is a megadiversity country (Mackinnon et al. 1996). Together with its lengthy history of cultivation of this economic mushroom, it might be that China is the origin of *Lentinula edodes* although we currently lack any fossil record. Our work has dealt with three provinces in continental China but is still an incomplete assessment of the biodiversity of this economically important mushroom. It does reveal, however, that this mushroom continues to evolve and diversify in nature so we can expect that proper protection of the remote habitats will maintain the genetic health of the native population of this fungus.

4 ACKNOWLEDGEMENTS

We thank The University Grants Committee of Hong Kong for supporting this study with an earmarked grant (CUHK4077/97M). We thank Mr. Z. M. Wang, Miss M. L. Yip, Miss Karen Cheung, Mr. Eric Leung and Mr. Ben Yeung for technical assistance. SWC is grateful to her family, especially parents, for their support.

REFERENCES

Anonymous 1998. *Chinese Medicinal Mycology*. Peking, China: Peking Medicinal University & China Kigua Medicinal University Joint Press. In Chinese.

Chakravarti, A. 1999. Population genetics - making sense out of sequence. *Nature Genetics* Suppl. 21: 56-60.

Chihara, G. 1993. Medical aspects of lentinan isolated from *Lentinus edodes* (Berk.) Sing. In S. T. Chang, J. A. Buswell & S. W. Chiu (eds), *Mushroom Biology and Mushroom Products*: 261-266. Hong Kong: The Chinese University Press.

Chiu, S. W., H. S. Kwan & S. C Cheng 1993. Application of arbitrarily-primed polymerase chain reaction in molecular studies of mushroom species with emphasis on *Lentinula edodes*. In S. T. Chang, P. G. Miles & J. A. Buswell (eds), *Culture Collection and Breeding of Edible Mushrooms*: 265-284. Philadelphia: Gordon & Breach Pub., Inc.

Chiu, S. W., A. M Ma, F. C Lin & D. Moore 1996. Genetic homogeneity of cultivated strains of shiitake (*Lentinula edodes*) used in China as revealed by the polymerase chain reaction. *Mycol. Res.* 100: 1393-1399.

Chiu, S. W., M. L. Ching, K. L. Fong & D. Moore 1998. Spent oyster mushroom substrate performs better than many mushrooms for removal of biocide pentachlorophenol. *Mycol. Res.* 102: 1553-1562.

Chiu, S. W., Z. M Wang, W. T Chiu, F. C. Lin & D. Moore 1999. An integrated study of individualism in *Lentinula edodes* in nature and its implication for cultivation strategy. *Mycol. Res.* 103: 651-660.

Felsenstein, J. 1989. PHYLIP – Phylogeny inference package (version 3.2). Cladistics 5: 164-166.

Felsenstein, J. 1993. *PHYLIP (Phylogeny Inference Package) version 3.5c*. Washington State, USA: Department of Genetics, University of Washington, Seattle.

Fox, H. M., J. Burden, S. T. Chang & J. F. Peberdy 1994. Mating-type incompatibility between commercial strains of *Lentinula edodes*. *Exp. Mycol.* 18: 95-102.

Fukuda, M., Y. F. Nakai, D. S. Hibbett, T. Matsumoto & Y. Hayashi 1994. Mitochondrial DNA restriction fragment length polymorphisms in natural populations of *Lentinula edodes*. *Mycol. Res.* 98: 169-175.

Hibbett, D. S., Y. Fukumasa-Nakai, S. Tsuneda & M. J. Donoghue 1995. Phylogenetic diversity in shiitake inferred from nuclear ribosomal DNA sequences. *Mycologia* 87: 618-638.
Hibbett, D. S., K. Hansen & M. J. Donoghue 1998. Phylogeny and biogeography of *Lentinula* inferred from an expanded rDNA dataset. *Mycol. Res.* 102: 1041- 1049.
Ho, K., Z. Hun & J. Yei 1994. *The Chinese Shiitake*. Shanghai, China: Shanghai Science & Technology Press. in Chinese.
Kimura, M. 1980. A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16: 111-120.
Kwan, H. S., S. W. Chiu, K. W. Pang & S. C. Cheng 1992. Strain typing in *Lentinula edodes* by polymerase chain reaction. *Exp. Mycol.* 16: 163-166.
Luo, X. C. 1998. Mushroom genetic resource, evaluation and utilization in China. In M. Lu, K. Gao, H. -F. Si & M. -J. Chen (eds), *Proceedings of the 98 Nanjing International Symposium on Science and Cultivation of Mushrooms*:142-152. Nanjing, China: JSTC-ISMS
Mackinnon, J., M. Sha, C. Cheung, G. Carey, X. Zhu & D. Melville 1996. *A Biodiversity Review of China*. Hong Kong: WWF International.
Mizuno, T. 1999. The extraction and development of antitumor-active polysaccharides from medicinal mushrooms in Japan. *Int. J. Med. Mush.* 1: 9-30.
Mori, K, Fukai, S. & Zennyoji, A. 1974. Hybridization of shiitake (*Lentinus edodes*) between cultivated strains of Japan and wild strains grown in Taiwan and New Guinea. *Mushroom Sci.* 9: 391-403.
Nicholson, M. S., B. A. Bunyard & D. J. Royse 1997. Phylogeny of the genus *Lentinula* based on ribosomal DNA restriction fragment length polymorphism analysis. *Mycologia* 89: 400-407.
Shimomura, N., K. Hasebe, Y. Nakai-Fukumasa & M. Komatsu 1992. Intercompatibility between geographically distant strains of shiitake. *Rept. Tottori Mycol. Inst. (Japan)* 30: 26-29.
Thorn, M. R. & D. J. Royse 1999. Partial β-tubulin gene sequences for evolutionary studies in the Basidiomycotina. *Mycologia* 91: 468-474.
Van Nieuwenhuijzen, B. 1998. Quality: keyword for the mushroom industry. *Mushroom J.* 579: 13-19.
Wang, N. L. 1998. Present condition and prospects of edible mushroom industry in China. *Edible Fungi of China* 17(5): 3. [In Chinese].
White, T. J., T. Bruns, S. Lee & J. Taylor 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White (eds), *PCR Protocols: A Guide to Methods and Applications*: 315-322. San Diego: Academic Press.
Yang, M. -S., C. - C. Chyau, D.-T. Horng & J.-S. Yang 1998. Effects of irradiation and drying on volatile components of fresh shiitake (*Lentinus edodes* Sing.). *J. Sci. Food Agric.* 76: 72-76.
Yoon, C. -S. & D. A. Glawe 1993. Association of random amplified polymorphic DNA markers with stromatal type in *Hypoxylon truncatum sensu* Miller. *Mycologia* 85: 369-380.
Yoon, C. -S., D. A. Glawe & P. D. Shaw 1991. A method for rapid small-scale preparation of fungal DNA. *Mycologia* 83: 835-838.
Zhang, S. & M. Lai 1993. The *Culture and Cultivation History of the Chinese Shiitake*. Shanghai, China: Shanghai Science & Technology Press . In Chinese.