

The development of the Fungal Resources Conservation System «One District, One Herbarium and Five Banks» and its application on Qinghai-Tibet Plateau*

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Biodiversity is closely related to human health and ecological environment. It has become very important due to its influence on the economic and political strategies of various countries. Fungal diversity is an important part of biodiversity and is of great significance in maintaining the balance of ecosystems. On the basis of fungal diversity studies the innovative conservation system «One district, one herbarium and five banks» was proposed and later applied on Qinghai-Tibet Plateau. In order to protect important fungal resources a conservation district of Yajiang Matsutake Reserve was established in Sichuan Province. To preserve the species, genetics and local natural fungal resources, the herbarium, the spawn bank, the viable tissue bank, the gene bank, the compound bank, and the comprehensive information bank were created, which resulted in the systematic protection of fungal diversity on Qinghai-Tibet Plateau and provided support for the sustainable use of fungi. Innovation and integration of the protection system of fungal resources and key technologies for sustainable utilization not only restore the ecological environment of important wild Fungal resources but also screen and cultivate new varieties of edible and medicinal fungi. It has been promoted and demonstrated and gave significant ecological, social and economic benefits.

Keywords: Fungi, slime mold, diversity, *Tricholoma matsutake*, edible and medicinal mushroom

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Развитие системы сохранения грибных ресурсов «Один район, один гербарий и пять банков» и ее применение на Цинхай-Тибетском плато

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*Сохранение биологического разнообразия приобрело в современном мире важное значение, как фактор, определяющий экономическое и политическое развитие отдельных государств, поскольку тесно связано с качеством жизни человека и стабильностью окружающей среды. Видовое разнообразие грибов – существенная часть общего биологического разнообразия, обеспечивающая устойчивое функционирование экосистем. На основании научных исследований для практического использования разработана инновационная система сохранения биологического разнообразия грибов под названием «Один район, один гербарий и пять банков», которую в Китае успешно реализовали в районе Тибетского Нагорья. С целью охраны уникальных грибных ресурсов, в уезде Яцзян, провинции Сычуань, был организован заповедник по воспроизводству грибов вида мацутаке (*Tricholoma matsutake*). Для того, чтобы сохранить в природе уникальные виды, исследованы их генетические особенности, природные комплексы среды обитания, оценены ресурсные запасы грибов местных видов, собран микологический гербарий, созданы банки мицелиальных культур, гермоплазмы, генетических источников, ценных метаболитов и базы данных с максимально полной информацией, которые обеспечивают систему защиты грибного разнообразия в пределах Тибетского Нагорья и способствуют поддержанию баланса между постоянным возобновлением грибных ресурсов и их экологически безопасной промышленной заготовкой. Инновационный и интеграционный характер системы охраны грибных ресурсов, а также базовые технологии возобновления и рационального использования крупнейших запасов дикорастущих грибов не только способствуют восстановлению экологической среды, но также позволяют отбирать в природе и вводить в культуру новые виды пищевых и лекарственных грибов. Внедрение системы охраны грибных ресурсов обеспечило высокие результаты в сферах экологии, общественной жизни и экономики.*

Ключевые слова: грибы, миксомицет, разнообразие, *Tricholoma matsutake*, пищевой и лекарственный гриб

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As an independent kingdom of eukaryotes, fungi have a large number of species. Hawksworth [1, 2, 3] estimated the number of fungal species in the world to be 1.5 million. Subsequently, Blackwell [4] established that the actual range of global fungal diversity was between 800,000 and 5.1 million species. With the advance of sequencing of large-scale environmental samples, the researchers believe that previous reports overestimated the fungal abundance by about 1.5 to 2.5 times [5]. Nonetheless, the global fungal number remains vast. However, only 100,000 species have been reported worldwide [6], which is less than 5% of the estimated number, and only 1% of those known species have been sequenced [7, 8]. At present, there are about 27,900 species recorded in China which belong to 3534 genera, 585 families, 192 orders, 56 classes, 15 phyla [9].

Fungal diversity is one of the important factors affecting the balance of the ecosystem. In addition to being an important decomposer of the earth's ecosystem, fungi also form close relationships with other organisms to realize carbon cycle and energy flow in the ecosystem, promote nutrient absorption of plants, enhance plant stress resistance and improve productivity [5, 10]. In addition, the diversity of edible and medicinal fungi is closely related to human health and environmental protection, and has become one of the main factors affecting the economic and political strategies of many countries. Currently, 1789 species of edible fungi and 798 species of medicinal fungi have been reported in China [11]. More than 100 species of fungi have been domesticated and cultivated, and 60% of them have been commercially produced. The industry of edible and medicinal fungi not only provided people with food and filled their nutritional needs, but also raised employment level and allowed for earning money [9].

Due to climate and field usage change, environmental pollution, nitrogen deposition, habitat loss and fragmentation, fungal diversity is under a threat that cannot be ignored, and some species have disappeared from the earth before being discovered [12, 13]. However, at a global, regional and local scale basic data such as fungal species and their gene sequences are still insufficient, and the influencing mechanism of fungal diversity is to be clarified, which makes the work on fungal diversity conservation extremely difficult [14].

Therefore, large-scale systematic collection, rapid and accurate identification of fungi are important prerequisites for study and protection of fungal diversity. A crucial component of biodiver-

sity protection is to establish a protection system of fungal resources.

The important tasks in implementing protection of fungal diversity include establishing fungal conservation centers, the mycological herbarium, the culture preservation center, the database of all kinds of information, and evaluation mechanisms of fungal diversity. The "One Conservation, One Herbarium and Five Banks" protection system of mycological diversity includes the Mycological conservation, the Mycological herbarium, the Spawn bank, the Viable tissue bank, the Compound bank, the Gene bank and the Comprehensive information bank to protect fungal resources regarding species, heredity and functions.

China has three ecological regions, namely: the eastern monsoon ecological, the northwest arid ecological and the Qinghai-Tibet alpine ecological regions. The Qinghai-Tibet plateau is the largest plateau with the highest average altitude in the world and is known as the "World's Third Pole". This region contains several typical ecological types: the alpine forests, the alpine meadow, the alpine desert and semi-desert. The special environment and physiognomy of the Qinghai-Tibet plateau ensures unique biological diversity. However, the extent of fungal resources existing within the Qinghai-Tibet plateau is still uncertain. With the accelerated development and commercial production of *Cordyceps sinensis*, matsutake and other rare fungi in recent years, fungal resources have been threatened and destroyed on Qinghai-Tibet plateau. In order to further promote the study and protect the fungal diversity on Qinghai-Tibet plateau from 2012 to 2015 our research group conducted dedicated applied research and implemented the "One District, One Herbarium and Five Banks" system for conservation of the fungal resources in Tibet.

Functional positioning of the "One District, One Herbarium and Five banks" fungal diversity protection system. After years of mycological researches and the promoting of the edible and medicinal fungal industry, academician Li Yu took the protection and sustainable utilization of fungal diversity as the basic foundation of the fungal resource protection system and established the operational framework of the "One District, One Herbarium and Five Banks" system.

"One District": In the conservation area important and rare fungi (mainly edible and medicinal fungi) are protected in situ;

"One Herbarium": the herbarium organizes, identifies and preserves all fungal specimens collected from the area;

"Five banks":

Spawn bank: separation, identification, succession and preservation of the fresh specimens;

Viable tissue bank: the samples of fruiting bodies are dried quickly to be preserved;

Gene bank: preservation of genomic DNA, ITS and other gene fragment information;

Compound bank: preserves the main chemical components, active components and spectral information of important edible and medicinal fungi;

Comprehensive information bank: preserves comprehensive information of specimens, including Latin nomenclature, classification status, collection information, photographic images, viable tissue, strain, geographical information (longitude, latitude, altitude) etc.

Based on traditional herbaria, spawn preservation centers and biological gene banks, integrated research has been conducted with the professional perspective of spawn resource classification to increase conservation areas, viable tissue libraries, compound libraries and comprehensive information databases. It should improve the function, the efficiency and the application of the spawn diversity protection system. China will play a positive role in biological protection, scientific and technological research and development, industrial promotion, personnel training, and promote the development and innovation of agriculture, biology, medicine and other industries.

Establishment of "One District, One Herbarium and Five Banks" system on Qinghai-Tibet plateau. The Qinghai-Tibet plateau has a complex topography. The population is small, many places such as the Yarlung Zangbo Grand Canyon, have high mountains and deep valleys, the greater part of the areas is almost uninhabited and it provides shelter for rare fungal species. However, the increase in collecting activities in some areas will impact the growth of rare mushrooms on Qinghai-Tibet plateau. Therefore, it is very important to strengthen initiatives to protect macrofungi in these areas. The following areas on Qinghai-Tibet plateau are the main collection areas based on their ecological types and vegetation distributions: Nyingchi, Qamdo, Nagqu, Shigatse, Shannan, Aba Tibetan Autonomous Prefecture, Ganzi Tibetan Autonomous Prefecture, Diqing Tibetan Autonomous Prefecture, Sanjiangyuan and Golmud.

Through field investigation, classification and identification of fungal resources in the representative areas of Qinghai-Tibet plateau, nearly 13,000 macrofungal specimens, belonging to 27 classes, 22 orders, 70 families and 273 genera

were obtained. Nearly 2,800 myxomycetes specimens belonging to 1 class, 5 orders, 8 families, 25 genera and 94 species were defined. Identification was conducted on cellular slime molds from 17 species, belonging to 1 class, 1 order and 2 families. On the basis of fungi diversity investigation, the conservation system of "One District, One Herbarium and Five Banks" of Qinghai-Tibet plateau flora resources was established for comprehensive protection of fungal species diversity, genetic diversity and ecological diversity.

The mycological conservation area. In terms of the selection and division of the mycological conservation areas, the priority of our research group was to select the conservation areas with the aim of species protection. At the same time, in the management of conservation areas, not only the population and quantity of protected species should be considered, but also other factors, such as local vegetation, ecosystems, endemic species, threatened species, economic and social conditions [15, 16].

From the beginning of 1980s, *Tricholoma matsutake* (S. Ito & S. Imai) has become the main source of income for Yajiang farmers. A large number of local people flocked to Songrong Mountain for fungal collection. However, due to the lack of biological knowledge on proper collection protocols, they destroyed the environmental conditions for further growth of *T. matsutake*. With the economic development, the Matsutake industry has a great potential. For a long time, the development of China's Matsutake industry was low, and it remained at that level of original acquisition, primary processing, and semi-finished products export. There is a serious lack of deep processing with high added value.

According to the characteristics of rare fungal resources on Qinghai-Tibet plateau, Sichuan Matsutake is mainly distributed in alpine valleys of 3000-500 m altitude, the main forest types are alpine oak forests, alpine pine and alpine mixed forests. Considering the geographical distribution, biological and habitat characteristics of *Tricholoma matsutake* resources, in August 2013 at the conference "China Matsutake Industry Development Summit" held in Chengdu, academician Yu Li signed a cooperation agreement on the conservation of matsutake resources with Gexigou Nature Reserve of Yajiang County, People's Government - "Wild Edible Mushroom Resource Conservation Cooperation Agreement". After that the Matsutake conservation area was built in Yajiang, Sichuan.

Combined with fungal resources research and market exploration, the requirements of collecting, purchasing and standardizing the harvesting of matsutake were put forward for the matsutake harvesting in conservation base, including the collection of matsutake, acquisition standards, acquisition requirements, transportation and preservation conditions of *T. matsutake*. Through contractual operation and closed off hillsides for matsutake conservation the forest ecosystem benefits have been significantly improved. According to the needs of protection management, perennial monitoring of major protected objects such as matsutake and their habitats, the basic data were obtained, laying the foundation for monographic scientific research, provided with a demonstration of sustainable conservation and development of Matsutake resources.

The Herbarium. Biological specimens are the basic materials for scientific research, it is a direct document reflecting the diversity of species. Species are the basic unit that constitutes the diversity of ecosystems, and it is also the main carrier of genetic diversity [17, 18]. In the historical development of human society, species are the important resources upon which natural productivity depends and there is a need to protect it.

The Qinghai-Tibet Plateau mycological herbarium is mainly composed of dried fruiting bodies of macrofungi and myxomycetes collected in this region. At present, from localities such as Tibet, Qinghai, Diqing Tibetan Autonomous Prefecture of Yunnan, Ganzi Tibetan Autonomous Prefecture of Sichuan, Aba Tibetan Autonomous Prefecture of Sichuan and Gannan Tibetan Autonomous Prefecture of Gansu, nearly 13,000 specimens of macrofungi and 2800 specimens of myxomycetes were collected.

The Qinghai-Tibet Plateau mycological herbarium was built in Jilin Agricultural University according to the characteristics and technical requirements of the specimens. This mycological herbarium is provided with specimen boxes, specimen cabinets, dryers, low temperature refrigerators (-80 °C), air conditioners, carbon dioxide fire extinguishers and other equipment. The purpose of these items is to prevent fire, excess moisture, insects penetration and to maintain constant temperature conditions for the preservation of specimens. The room where the specimens are to be kept is equipped with air conditioners, liquid nitrogen fire extinguishers and other equipment. The room temperature is kept at 20-23 °C and the humidity is about 40%. The specimens are placed in sterile whirl-pack plastic bags together with the

desiccant, and the bags are put into the specimen boxes. These specimen boxes are labeled with the basic information such as the species name, specimen number, the place and date of collection, as well as the name of the collector. These samples are stored in specimen cabinets, chronologically. The status of specimens should be regularly checked.

Spawn bank. Edible and medicinal fungal spawns are important biological resources, which are the basis for the implementation of production and scientific research. Good spawns are easily degraded especially after long-term use; it leads to the need for preservation of these spawns for sustained usage.

Wild edible and medicinal fungi in Qinghai-Tibet plateau were collected according to the characteristics of high development potential, high economic value, prominent medicinal efficacy, tissue isolation and spawn. The rare edible and medicinal fungi in this region were obtained for breeding and preservation.

The Qinghai-Tibet Plateau spawn bank is equipped with a low-temperature warehouse of 4°C, an ultra-low temperature warehouse of -80°C and a liquid nitrogen storage bank. The fruiting bodies of edible and medicinal fungi were classified in the field, purified cultured and species identification under the laboratory conditions. According to the biological characteristics of these spawns, they were inoculated into suitable media and cultured under appropriate conditions. After growing adequately, the fruiting bodies of edible and medicinal fungi were preserved at 4°C and sub-cultured regularly. Some mycelia or spores of rare fungi were stored in freezing tubes containing glycerol, in freezers at - 80°C or in liquid nitrogen tanks. At present, there are 426 spawns in Qinghai-Tibet plateau spawn bank, including many rare edible and medicinal spawn.

Viable tissue bank. Population genetic structure variation of organisms is the result of a combination of factors such as population evolution, distribution, and breeding, which reflects the adaptability and evolutionary potential of population units, and is also related to the formulation of strategies and measures for species protection and rejuvenation [19]. Efficient access to genetic information of important biological populations is the necessary basis for this work. Viable tissue can preserve the genetic information of fungal species quickly and efficiently.

After collecting fresh fruiting bodies, 1~2 cm dissected specimens, wrapped in paper with good air access should be dried in 2 ml

centrifugal tube containing discolored silica gel. According to the color changes of those silica gel, the silica gel should be replaced for rapid drying of materials. During the time of fungi field collection on Qinghai-Tibet plateau, nearly 3000 living tissues were obtained. The genome information of these samples was effectively preserved, which would provide basic materials for the following research.

Gene bank. Mycology genomic DNA is one of the most important genetic materials. High-quality DNA should meet certain standards in genomic integrity, purity, concentration and content. The length of genomic DNA used for preservation should be greater than 15kb; purity should be $1.8 \leq A_{260}/A_{280} \leq 2.0$, showing a single bright band in gel electrophoresis detection; genomic DNA concentration must not be less than $100 \text{ ng} \cdot \mu\text{L}^{-1}$, volume no less than $100 \mu\text{L}$; each sample stored in 3 tubes, stored in -80°C cryogenic refrigerator for extended periods of time, and the quality of samples regularly sampled at random, and replacement samples are updated in time.

Nowadays, the DNA barcode is an important reference for taxonomy and molecular biology. ITS rDNA can clarify the resolution of fungal species up to 72%. It is the single DNA fragment with the highest resolution of fungal species. ITS was officially recommended as the preferred DNA barcode for fungi at the 4th International Conference on Life Barcode held in Adelaide, Australia, in 2011. The ITS1-5.8S-ITS2 region was amplified by ITS universal primers [20, 21]. For ITS barcode amplification of important fungal species, it is necessary to maximize the coverage of the survey area in different individual collection sites of the same species during field investigation. According to the requirements of the International Barcode of Life Project (iBOL Project <http://ibol.org/phase1/>) and the regional ecological environment differences [22], 6-12 individuals of each species are selected for DNA extraction and ITS barcode amplification. Preserved voucher specimens are maintained in the herbarium and with established species identification reference databases help to obtain more complete population genetic information [23, 24].

Nearly 7000 genomic DNA fragments and 6000 ITS ribosome fragments were obtained from fungal specimens collected on Qinghai-Tibet plateau. The gene bank of important fungi collected from this region was established in population and above genus levels, including *Ophiocordyceps sinensis* (Berk.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora, *Tricholoma matsutake* (S. Ito & S. Imai) Singer, *Floccularia luteovirens* (Alb.

& Schwein.) Pouzar, *Boletus*, and those corresponding sequence tag information bank.

Compound bank. Fungi is one of the important sources of active natural products, and their metabolites play an important role in many different drug researches and developmental strategies. The discovery of new species of fungi and the development of new compounds had great significance to the screening of active ingredients, the discovery of lead compounds and the evaluation of the nutritional quality of edible and medicinal fungi.

Compound bank includes pre-isolated active sites extracted from edible and medicinal fungi samples, monomer compounds, and corresponding spectral data information. Pre-isolation active sites were mainly filtrated and crude polar extracts of mycelia, wild or cultivated fruiting bodies from liquid fermentation of edible and medicinal fungi [25]; monomer compounds included pure natural products with purity of more than 80% and no repetitive structure [26]; spectral data were mainly ^{13}C -NMR, ^1H -NR, DEPT, HMBC, HMQC, H-H COSY corresponding to monomer compounds and GC-MS data of pre-separated lipid-soluble components.

The 986 kinds of lipid-soluble components, more than 70 monomer compounds and their related spectral data from nearly 20 species of rare fungi, such as *F. luteovirens*, *Sarcodon imbricatus* (L.) P. Karst., *T. aurantialba*, *O. sinensis*, collected in Qinghai-Tibet Plateau were obtained.

Comprehensive information bank. In order to research and utilize the fungal resources more effectively, a comprehensive database of fungal resources was established by means of computer database technology. Information in the database includes the following: collection number, the date of collection, name of the collector, photographic images, viable tissue, strain, Chinese name, Latin name, geographic information (Longitude, latitude, altitude), names of collecting location, corresponding genetic information and compound information. Information can be quickly searched according to the collection number or Latin name of the specimen. Microsoft Office Access database was used in the information bank on Qinghai-Tibet Plateau.

Prospect. At present, the total number of fungal resources in China is still uncertain. Mushrooms of economic importance as well as rare mushrooms are greatly endangered and destroyed because their living conditions are affected by excessive artificial collection and production. Research on fungal resources in China is in the development stage, there are still many unknown areas to explore, and technical problems need to

be addressed and overcome. The authoritative database of fungal resources can provide strong support for the study of fungal resources, and promote the research in many aspects. These include: the pattern of fungal resources, planning for conservation of fungal resources, its response to global changes, prediction of invasive exotic species, effectively monitoring the flow and protection of fungal germplasm resources, which also play an important role in the study of fungi and provides important support for the study of fungal resources.

In the study of Chinese fungal resources, the implementation of effective fungal resource conservation mechanisms is still a weak link, and needs more infrastructure development. The ecosystems of China are very rich, including the main types of terrestrial ecosystems on the earth, such as forests, shrubs, grasslands, meadows, deserts, and tundra. By the end of 2015, China had 2740 nature reserves with a total area of about 1.47 million km²: there were 525 nature reserves with wild animals as the main protection type (including 109 national protected areas), covering an area of 387,000 km²; 156 nature reserves with wild plants as the main protection type (including 19 national protected areas), and 17,000 km² national protected areas. The nature reserves with fungi as the main object for protection is countless.

In addition, research on conservation biology based on biodiversity still lacks information mechanism and theoretical systems, research methods are also controversial [27, 28, 29]. At present, the work of biodiversity assessment and conservation mainly focuses on animals and plants [30, 31], and there is a lack of theoretical research on the distribution pattern of fungal biodiversity, and the implementation of the biodiversity protection system combined with it is also to be explored in many ways.

The establishment of the “One District, One Herbarium and Five Banks” conservation system

for fungal resources is different from the traditional researches at home and abroad, which is also different from the traditional mycological herbarium (collection of specimens for resource investigation or separation of preserved strain for application), it includes the establishment of the conservation, the viable tissue bank, the gene bank, the compound bank, and the information bank. Fungal resources are protected while these species diversity is preserved in ecological function of population, genetic information, and chemical information.

Meanwhile, the Metabarcoding technology has been used to improve the species diversity of fungi in the forests of the Eastern Qinghai-Tibet Plateau. A lot of potential species have been found, which is not only an important supplement to the information of specimens but also fundamental information for the study of fungal diversity on Qinghai-Tibet Plateau. At the same time, it also provided a reference for improving the methods of investigation of fungal resources and the construction of the protection system.

On the other hand, the research results of the conservation system “One District, One Herbarium and Five Banks” of fungal resources on Qinghai-Tibet Plateau provided not only the data for fungal diversity and the influencing factors of these changes, but also a firm foundation for a series of key technical researches such as domestication of rare strains, production of active substances and development of functional genes [32, 33, 34]. It provided the advanced theoretical basis, technical means and scientific methods for the development of edible and medicinal fungal industry on Qinghai-Tibet Plateau, and realized the sustainable development of a variety of rare and common fungi in this region. The developmental direction of conservation and sustainable utilization of fungal resources is supported by the preservation and utilization of fungal resources.

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