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Full Length Research Paper

Application of SRAP in the genetic diversity of *Tricholoma matsutake* in northeastern China

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Tricholoma matsutake **is an ectomycorrhizal (ECM) fungus that produces economically important mushrooms. The study firstly applied SRAP technique into genetic diversity of *T. matsutake.* A total of 129 strains from 13 geographical locations in northeastern China, were amplified by using selected 12 primer pairs. The polymorphic band number amplified by each primer pair ranged from 7 to 13. In total 154 bands were observed, of which 118 were polymorphic (76.62%). Abundant genetic variation was detected within individual populations. Dongning maintained a higher genetic diversity while Hunchun was lower. The analyses found a significant positive correlation between genetic distance and geographical distance and no correlation between genetic distance and altitudinal differences among populations. Based on the UPGMA cluster diagram, the 13 populations may be divided with the genetic distance of 0.035 into three groups.**

Key words: Genetic diversity, *Tricholoma matsutake*, fungi, sequence related amplified polymorphism (SRAP).

INTRODUCTION

The ectomycorrhizal fungus *Tricholoma matsutake* is one of the most delicious and valuable edible mushrooms in Asia, especially in Japan, Korea, and China (Hall et al., 2003). Through northeastern China, *T. matsutake* grows widely in coniferous trees, especially *Pinus densiflora* forests, and sometimes it can also be found in *P. thunbergii, P. pumila.* While in southwestern China, it grows in broad-leaved forests mainly consisting of *Castanopsis* spp. and *Quercus* spp. (Wang et al., 1997). In the past century, because of deforestation and infestation by the pinewood nematode (*Bursaphelenchus xylophilus*), the host plant populations of *T. matsutake, P. densiflora* declined rapidly in Japan (Wang and Hall, 2004). As a result, the annual harvest of *T. matsutake* in Japan has been much lower than it used to be in the early 20th century. Because artificial cultivation has not been developed for any of the matsutake mushrooms, including *T. matsutake*, to satisfy its domestic demand, Japan imports about 3000 tons of *T. matsutake* annually, mostly from Pacific North America, Korea and China. As an important production region, Heilongjiang and Jilin in northeastern China accounts for 39% of the total amount of the exported *T. matsutake* of China.

Although *T. matsutake* mushrooms have a high commercial value and a very important role in non-timber forest products, very little is known about the relationships among geographical populations, either in Japan or elsewhere. A study has examined the relationship between genetic distance and geographical distances in *T. matsutake* (Chapela and Garbelotto, 2004). In this study, seven strains of *T. matsutake*, three from China and one each from Korea, Japan, France, and Morocco, were examined. A significant positive correlation was found between their genetic dissimilarity based on genotypes identified using amplified fragment length polymorphisms (AFLP) and their geographical distances. Sha et al. (2007) sequenced the internal transcribed spacer (ITS) and the intergenic spacer (IGS) regions of the rDNA, and found no polymorphism among 56 fruiting bodies collected from 13 counties in Yunnan.

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Abbreviations: ECM, Ectomycorrhizal; **SRAP,** sequence-related amplified polymorphism; **AFLP,** amplified fragment length polymorphisms; **ITS,** internal transcribed spacer; **IGS,** intergenic spacer; **PCR,** polymerase chain reaction; **FST,** mean level of genetic differentiation.

Table 1. Geographic distributions of samples in northeastern China.

Sampling site	Latitude (North)	Longitude (East)	Altitude (m)	Sample size (N)	Year
Hailin	44.65	129.15	421	3	2005
Dongjingcheng	43.58	129.34	453	15	2007
Jidong	45.12	131.27	358	12	2006
Muling	44.97	130.65	534	6	2008
Dongning	43.24	130.59	296	16	2007
Wangqing	43.32	129.78	689	9	2008
Baijin	42.48	129.37	515	10	2007
Fuyu	41.23	127.54	752	9	2006
Fuxing	42.16	129.07	810	13	2005
Longmen	42.73	129.18	712	11	2006
Hunchun	42.86	130.34	605	10	2005
Changxing	43.17	128.95	783	7	2007
Shimen	43.28	128.78	653	8	2006
Total sample	41.23-45.12	127.54-131.27	296-810	129	2005-2008

High polymorphism could be detected, however, in strains from Japan using a specific PCR primer. Sequence-related amplified polymorphism (SRAP) is a new PCR-based marker, firstly raised by Li and Quiros, (2001). SRAP was similar to RAPD, but it was a preferential random amplification of coding regions in genome. SRAP had been applied extensively in genetic diversity analysis (Ferriol et al., 2003) and comparative genetics (Lin et al., 2004) of different species. However, up to now, the SRAP molecular marker had not been used to examine the patterns of genetic variation of *T. matsutake* from within and between populations. In this work, PCR–SRAP marker was adopted to study the genetic variation present in 129 strains from 13 geographical regions from northeastern China. The results will contribute to the conservation and sustainable utilization of *T. matsutake* of northeastern China.

MATERIALS AND METHODS

Sporocarp sampling

Sporocarps were collected during fruiting periods, from early September until the end of October of 2005 to 2008. All natural samples of *T. matsutake* were collected from forests consisting of *P. densiflora,* as the dominant species (with a frequency higher than 85%), *Quercus mongolica, Betula davurica* and shrub communites with *Rhododendron davuricum*, *Philadelphus schrenkii*. These fruiting bodies were collected from thirteen sites of northeastern China, including five in Heilongjiang and eight in Jilin. The investigated region stretched about 200 km from east to west and about 400 km from south to north, with an altitude span from 296 m above sea level in Dongning to 810 m in Fuxing. The counties, where the sampling sites were located, are shown in Table 1. A total of 129 fruiting bodies were collected, processed and stored at -70℃. The fruiting bodies were typically distributed from about 10 m of each other to as far as about 1 km from each other. These specimens were identified based on their macro- and micro morphological characteristics and confirmed based on their sequences at the internal transcribed spacer (ITS) region of the ribosomal DNA.

DNA isolation

Crude genomic DNA was extracted from sporocarps using a modified cetyl-trimethylammonium bromide (CTAB) method (Lian et al., 2003). The final DNA was suspended in 50 ml TE buffer and stored at -20℃.

SRAP-PCR amplification

A total of 132 different combinations of primers were employed using 11 forward and 12 reverse primers (Table 2). About two thirds of them could produce clear bands, but considering the polymorphism and reproducibility, 12 combinations were chosen in the following study (Table 3). The thermal cycling profile was 5 min of pre-denaturing at 94℃, followed by five cycles of 1 min of denaturing at 94℃, 1 min of annealing at 35℃ and 1 min of extension at 72℃, then 35 cycles of 1 min at 94℃, 1 min at 50℃ and 1 min at 72℃, and a final extension step of 7 min at 72℃. The PCR mixture consists of 5 ng of DNA template, 1.0 µM of each primer, 200 µM of dNTP each, 2.5 mM of $MgCl_2$, 1 × PCR buffer, and 1U Taq DNA polymerase in a total volume of 25 µl. The amplification was performed in an applied biosystems 9800 and negative controls were run at each step in order to ensure the reliability of the genotype. The PCR products were separated on 6% denaturing polyacrylamide gel (acrylamide: bisacrylamide = 19:1) and SRAP bands were stained using silver sequence TM DNA staining reagents (PROMEGA).

Data analysis

Amplified bands were scored 1/0 as presence/absence of homologous bands for all samples. The resulting presence/absence data matrix was analyzed using POPGENE version 1.32 (Francis and Yang, 2000) to estimate the level of genetic diversity. The following genetic diversity parameters including the percentage of polymorphic bands (PPB), effective number of alleles (Ne), Shannon's information index (I) and Nei's gene diversity (H) were obtained at both species level and population level (Nei, 1987). To test for isolation by distance, genetic distances and geographical

Table 2. The forward and reverse SRAP primer combinations used in this study.

Forward primers (5'–3')	Reverse primers (5'–3')
Me1 TGAGTCCAAACCGGATA	Em1 GACTGCGTACGAATTAAT
Me2 TGAGTCCAAACCGGAGC	Em2 GACTGCGTACGAATTTGC
Me3 TGAGTCCAAACCGGAAT	Em3 GACTGCGTACGAATTGAC
Me4 TGAGTCCAAACCGGACC	Em4 GACTGCGTACGAATTTGA
Me5 TGAGTCCAAACCGGAAG	Em5 GACTGCGTACGAATTAAC
Me6 TGAGTCCAAACCGGTAG	Em6 GACTGCGTACGAATTGCA
Me7 TGAGTCCAAACCGGACA	Em7 GACTGCGTACGAATTATG
Me8 TGAGTCCAAACCGGTGT	Em8 GACTGCGTACGAATTAGC
Me9 TGAGTCCAAACCGGTTG	Em9 GACTGCGTACGAATTACG
Me10 TGAGTCCAAACCGGCTA	Em10 GACTGCGTACGAATTCTG
Me11 TGAGTCCAAACCGGAGG	Em11 GACTGCGTACGAATTCAG
	Em12 GACTGCGTACGAATTCCA

Table 3. The polymorphism from 12 SRAP primer combinations.

Primer combinations	Total number of bands	Number of polymorphic bands	Percentage of polymorphic loci (%)
Me1/ Em12	15	12	80.0
Me2/ Em2	12	10	83.3
Me3/ Em3	10	8	80.0
Me3/ Em5	14	9	64.3
Me3/ Em6	12	8	66.7
Me4/ Em6	11	7	63.6
Me4/ Em12	15	12	80.0
Me5/ Em12	13	11	84.6
Me7/ Em4	11	9	81.8
Me8/ Em4	13	10	76.9
Me10/ Em3	16	13	81.3
Me10/ Em12	12	9	75.0

distances among sites were analyzed with Mantel tests using GENAIEX, version 5 (Peakall and Smouse, 2001). Weir and Cockerham (1984) estimated the mean level of genetic differentiation (FST) using GENEPOP, version 3.4. The phylogenetic tree was constructed using a UPGMA method in Mega 2. Finally, analysis of molecular variance (AMOVA; Excoffier et al., 1992) was conducted to estimate the relative contributions of local and regional geographical separations to the overall samples in northeastern China.

RESULTS

Polymorphism and genetic diversity of *T. matsutake* populations

A total of 129 samples from 13 sites of northeastern China were amplified by using selected 12 different SRAP primer combinations. The band number amplified by each pair of primers ranged from (Me3/ Em3) 10 to (Me10/Em3) 16, with an average of 12.8. A total of 154 bands were observed, among which 118 were polymorphic (76.62%), ranging between 7 (Me4/Em6) and 13 (Me10/Em3) per primer combination, with an average of 9.83 bands per primer set. The percentage of polymorphism for each primer combination varied from 63.6% (Me4/Em6) to 84.6% (Me5/Em12; Table 3). The size of scored bands ranged from 200 to 2000 bp.

Genetic diversity data for 13 populations of *T. matsutake* based on SRAP markers are summarized in Table 4. The percentage of polymorphic loci (PPB) of the 13 *T. matsutake* populations ranged from 33.33% (Fuyu and Hunchun) to 66.67% (Dongning). The obtained effective number of alleles (Ne) ranged from 1.1559 (Hunchun) to 1.2487 (Dongning). Nei's gene diversity index (H) ranged from 0.1012 (Hunchun) to 0.1690 (Dongning). Shannon's information index (I) ranged from 0.1451 (Hunchun) to 0.2252 (Dongning).

Genetic variation among geographical populations

The authors' analyses identified a range of FST values

Table 4. Genetic diversity of 13 *T. matsutake* populations from northeastern China.

Geographical population	PPB (%)	Ne	H	I
Hailin	50.00	1.2107	0.1310	0.1991
Dongjingcheng	41.67	1.1832	0.1223	0.1715
Jidong	58.33	1.2244	0.1589	0.2067
Muling	41.67	1.1751	0.1168	0.1665
Dongning	66.67	1.2487	0.1690	0.2252
Wangqing	41.67	1.1858	0.1291	0.1783
Baijin	50.00	1.2067	0.1335	0.1922
Fuyu	33.33	1.1564	0.1023	0.1469
Fuxing	58.33	1.2289	0.1593	0.2032
Longmen	50.00	1.2175	0.1217	0.1919
Hunchun	33.33	1.1559	0.1012	0.1451
Changxing	41.67	1.1743	0.1139	0.1628
Shimen	50.00	1.1823	0.1123	0.1751

PPB, Percentage of polymorphic loci; Ne, the effective allele number; H, Nei's gene diversity; I, Shannon's information index.

between pairs of local populations. The lowest value (0.007) was found between Fuxing and Longmen, while the highest (0.174) was found between Jidong and Changxing (Table 5). Overall, the mean FST value was about 0.10. About 10% of the gene diversity was due to geographical separations between pairs of populations. Analysis of molecular variation analysis further revealed significant genetic variation ($p<0.01$) among and within 13 populations of *T. matsutake*. Of the total genetic variation, the main component (70.3%) is contributed to by the within population level, and the rest (29.7%) existed among populations (Table 6).

The relationship between genetic distance and geographical parameters

The results from the Mantel tests are shown in Figure 1. The test showed a significant positive correlation between genetic distance and geographical distance among the analyzed populations ($p = 0.013$). Based on the UPGMA cluster diagram, the 13 populations may be divided into three groups with the genetic distance of 0.035 (Figure 2). One group included Hailin, Wangqing, Fuxing, Fuyu, Longmen and Hunchun; another group, Baijin, and Changxing; the third group, Dongjingcheng, Jidong, Mulin and Dongning.

DISCUSSION

SRAP maker is more frequently used in the genetic study of vegetables and crops and rarely in the study of fungi. Sun et al. (2006) applied SRAP marker to analyze 31 different Ganoderma strains genetic diversity. In this study, the novel PCR-based SRAP marker had been successfully used in the research of genetic diversity of *T. matsutake.* The results indicated that SRAP marker was suitable for the molecular characterization and the investigation of phylogenic relationships in *T. matsutake* and proved that the use of SRAP approach was more efficient to examine the genetic diversity in basidiomycetes.

The authors analyzed a large number of geographical populations of the ectomycorrhizal mushroom *T. matsutake* from northeastern China. The results identified significant genetic variation within and between populations. Overall, these populations showed relatively low but significant genetic differentiation. Among the 13 populations, Dongning (PPB = 66.67%; Ne = 1.2487; H = 0.1690; I = 0.2252) maintains a higher genetic diversity than the others. In contrast, the level of polymorphism and genetic diversity of the Hunchun (PPB = 33.33%; Ne = 1.1559; H = 0.1012; I = 0.1451) was found to be lower than other populations (Table 4). In addition, the amount of differentiation varied between populations, with the level of differentiation correlated to some extent to geographical distances separating the populations, which is consistent with results from a previous study using different samples (Chapela and Garbelotto, 2004).

The analyses identified a range of FST values between pairs of local populations. The lowest value (0.007) was found between Fuxing and Longmen, while the highest (0.174) was found between Jidong and Changxing. The FST values observed between geographical populations of *T. matsutake* are similar to those reported in several basidiomycete species. For example, between regional populations of *Agaricus bisporus*, the FST values were found ranging between 0.019 and 0.076 (Xu et al., 2005). In *P. ferulae* and *P. eryngii,* 19 and 6 regional populations from Italy showed FST values of 0.45 and 0.10, respectively, for these two species (Urbanelli et al., 2003). In *T. matsutake,* Chapela and Garbelotto (2004) identified that

Table 5. Pairwise FST values between geographical populations of *T. matsutake* from northeastern China.

Pop ID	Shimen	Hailin	Dongjingcheng	Jidong	Muling	Dongning	Wangqing	Baijin	Fuyu	Fuxing	Longmen	Hunchun
Hailin	0.127											
Dongjingcheng	0.088	0.031										
Jidong	0.110	0.055	0.099									
Muling	0.101	0.052	0.048	0.042								
Dongning	0.033	0.065	0.096	0.077	0.051							
Wangqing	0.048	0.038	0.073	0.044	0.099	0.076						
Baijin	0.043	0.083	0.101	0.111	0.123	0.089	0.049					
Fuyu	0.051	0.047	0.084	0.065	0.087	0.038	0.075	0.064				
Fuxing	0.068	0.076	0.091	0.073	0.091	0.033	0.066	0.019	0.055			
Longmen	0.052	0.092	0.089	0.147	0.079	0.042	0.098	0.068	0.067	0.007		
Hunchun	0.074	0.087	0.096	0.087	0.071	0.049	0.035	0.065	0.086	0.082	0.065	
Changxing	0.086	0.105	0.096	0.174	0.065	0.098	0.031	0.086	0.075	0.009	0.011	0.073

Table 6. Analysis of molecular variation for SRAP data of *T. matsutake.*

Source of variation	d.f.	SS	MS	Est. var.	Total variation (%)	p
Among populations	12	101.763	8.156	0.663	29.7	0.010
Within populations	117	153.839	1.571	1.571	70.3	0.010
Total	129	255.602	9.727	2.234		

d.f., degree of freedom; SS, sum of squared observations; MS, mean of squared observations; Est. var., estimated variance.

among the seven strains they analyzed, they found a positive correlation between their pair wise genetic dissimilarity and geographical distances. Those seven strains were far apart from each other with a distance of thousands of kilometers. However, because of the small sample sizes (mostly only one strain from each country or geographical area), the level of genetic differentiation among geographical populations could not be assessed. At present, little is known about the genetic relationships of most ectomycorrhizal fungal populations from the 100 km ranges.

At present, in order to satisfy the demands of Japanese consumers, virtually all *T. matsutake* mushrooms are picked prematurely before the veils of the mushrooms are ruptured to release spores, which affects seriously the reproducibility of the mycelia and the spores and leads to the decreasing amount of *T. matsutake.* As a result, management plans should enforce the notion that at each site, a certain number of mushrooms must be allowed to mature and sporulate so as to allow future reproduction. Aside from better management decisions based on these observations, the authors believe that their population genetic study can also contribute to improved management strategy on sustainable resource utilizations of *T. matsutake* in northeastern China. It is well known that *T. matsutake* of northeastern China exists mainly in symbiosis with *P. densiflora*. However, the major host plant is *Castanopsis* spp. and *Quercus* spp. in southwestern China *T. matsutake*. Currently, whether or not such differences in host plants influence genetic differentiation among different *T. matsutake* populations is an open question. Further study is needed to address this

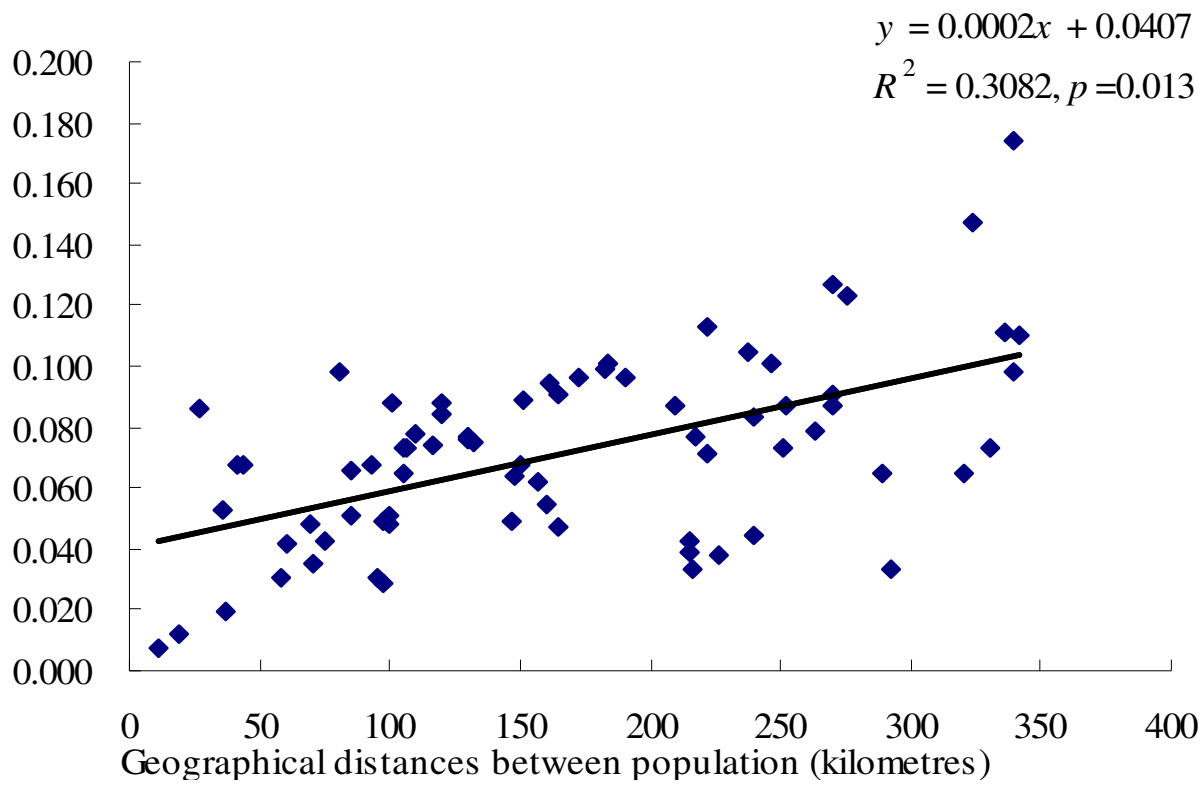

Figure 1. Results from Mantel tests between genetic differences and geographical distances among populations. The X-axis represents the geographical distance parameter and the Y-axis represents Nei's genetic distances between populations.

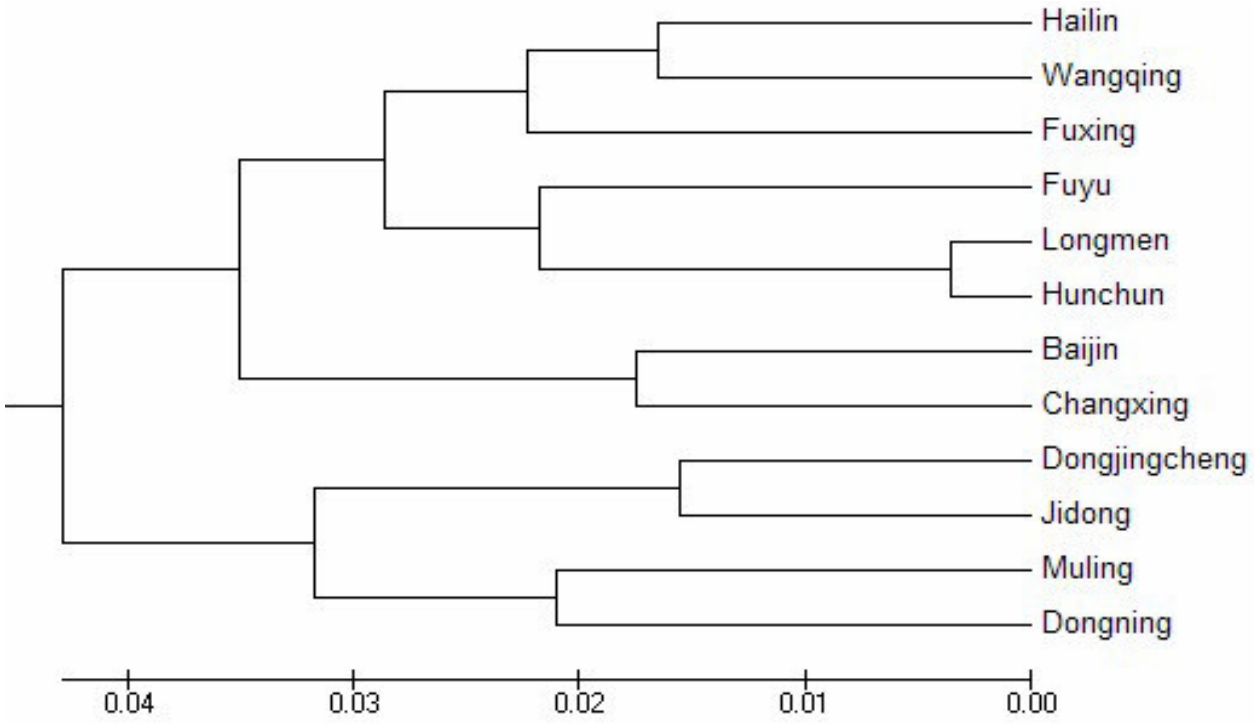

Figure 2. Genetic relationships among 13 *T. matsutake* populations.

issue.

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REFERENCES

Chapela IH, Garbelotto M (2004). Phylogeography and evolution in matsutake and close allies inferred by analyses of ITS sequences and AFLPs. Mycologia, 96: 730-741.

Excoffier L, Smouse PE, Quattro JM (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics, 131: 479-491.

Ferriol M, Picó B, Nuez F (2003). Genetic diversity of a germplasm collection of Cucurbita pepo using SRAP and AFLP marker. Theor. Appl. Genet. 107: 271-282.

Francis CY, Yang RC (2000). Popgene version 1.32. http// www.ualberta.ca/_fyeh/index.htm.

Hall IR, Yun W, Amicucci A (2003). Cultivation of edible ectomycorrhizal mushrooms. Trends Biotechnol. 21: 433-438.

Li G, Quiros CF (2001). Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theor. Appl. Genet. 103: 455-461.

Lian CL, Oishi R, Miyashita N, Nara K, Nakaya H, Wu B, Zhou Z, Hogetsu T (2003). Genetic structure and reproduction dynamics of Salix reinii during primary succession on Mount Fuji, as revealed by nuclear and chloroplast microsatellite analysis. Mol. Ecol. 12: 609-618.

Lin ZX, Zhang XL, Nie YC (2004). Evaluation of application of a new molecular marker SRAP on analysis of segregation population and genetic diversity in cotton. Acta Genet. Sinica. 31: 622-626.

Nei M (1987). Molecular evolutionary genetics. New York, USA: Columbia University Press.

Peakall R, Smouse PE (2001). GENAIEX V5: genetic analysis in Excel. Population genetic software for teaching and research. Canberra, Australia:AustralianNationalUniversity,http://www.anu.edu.au/BoZo/GenAIEx/.

Sha T, Zhang HB, Ding HS (2007). Genetic diversity of *Tricholoma matsutake* in Yunnan Province. Chinese Sci. Bull. 52: 1212-1216.

Sun SJ, Gao W, Lin SQ (2006). Analysis of genetic diversity in Ganoderma population with a novel molecular marker SRAP. Appl. Microbiol. Biotechnol. 72: 537-543.

Urbanelli S, Della Rosa V, Fanelli C, Fabbri AA, Reverberi M (2003). Genetic diversity and population structure of the Italian fungi belonging to the taxa Pleurotus eryngii (DC.Fr.) Quel and *P. ferulae* (DC.Fr.) Quel. Heredity, 90: 253-259.

Wang Y, Hall IR (2004). Edible ectomycorrhizal mushrooms: challenges and achievements. Can. J. Bot. 82: 1063-1073.

Wang Y, Hall IR, Evans LA (1997). Ectomycorrhizal fungi with edible fruiting bodies. I. *Tricholoma matsutake* and related fungi. Econ. Bot. 51: 311-327.

Weir BS, Cockerham CC (1984). Estimating F-statistics for the analysis of population structure. Evolution, 38: 1358-1370.

Xu J, Cheng M, Tan Q, Pan Y (2005). Molecular population genetics of basidiomycete fungi. In: Evolutionary Genetics of Fungi (ed. Xu J). Horizon Biosciences, Norfolk, UK. pp. 221-252.