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Recombination and genetic differentiation among natural populations of the ectomycorrhizal mushroom *Tricholoma matsutake* from southwestern China

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Abstract

Effective conservation and utilization strategies for natural biological resources require a clear understanding of the natural populations of the target organisms. *Tricholoma matsutake* is an ectomycorrhizal mushroom that forms symbiotic associations with plants and plays an important ecological role in natural forest ecosystems in many parts of the world. It is also an economically very important gourmet mushroom. Because no artificial cultivation is available, natural populations of this species are under increasing threats, primarily from habitat disturbance and destruction. Despite its economical and ecological importance, little is known about its genetics and population biology. Here, using 14 polymerase chain reaction–restriction fragment length polymorphism markers, we analysed 154 strains from 17 geographical locations in southwestern China, a region where over 25% of the global *T. matsutake* harvest comes from. Our results revealed abundant genetic variation within individual populations. The analyses of gene and genotype frequencies within populations indicated that most loci did not deviate from Hardy–Weinberg equilibrium in most populations and that alleles among loci were in linkage equilibrium in the majority of the local populations. These results are consistent with the hypothesis that sexual reproduction and recombination play an important role in natural populations of this species. Our analyses indicated low but significant genetic differentiation among the geographical populations, with a significant positive correlation between genetic distance and geographical distance. We discuss the implications of our results to the ecology and resource management of this species.

Keywords: biodiversity, conservation, fungi, matsutake mushroom, PCR-RFLP, SNP

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Introduction

Fungi play pivotal roles in human and animal health, agriculture, biotechnology, and forestry. They are essential components of all natural terrestrial ecosystems. In forest ecosystems, fungi are commonly found as decomposers on forest floors, as commensal endophytes or pathogens on plant stems, roots, leaves or fruits, and in or on plant roots as symbionts. The symbiotic association between fungi and plant roots is called mycorrhizae. The fungal mycelia around the roots help plants obtaining essential phosphate and minerals from the soil. The mycorrhizae not only help plants grow but also contribute to plants' disease resistance and drought tolerance (Brundrett 2004). Based on their structural associations, mycorrhizae are divided into two groups, endomycorrhizae and ectomycorrhizae. Almost all tree species form ectomycorrhizal associations with fungi that belong to divisions Zygomycota, Ascomycota, or Basidiomycota (Brundrett 2004). Among species in Basidiomycota that form ectomycorrhizal associations with plant roots, many produce mushrooms that are collected as sources of exotic and highly prized food for humans (Wang & Hall 2004).

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Tricholoma matsutake is an ectomycorrhizal basidiomycete that produces economically important edible mushrooms commonly known as 'matsutake'. It is predominantly associated with pine forests in the Northern Hemisphere but has been found to be associated with oaks and other tree species in southwestern China (Nakayama & Nakanishi 2004). Though morphologically indistinguishable, the difference in their associated dominant host tree species has been used as an ecological parameter to separate the matsutake mushrooms from southwestern China into two closely related species, *T. matsutake* and *Tricholoma quercicola* (Zang 1990) (synonym *T. zangii* Z.M. Cao *et al*. (2003), corresponding to forest habitats dominated by pines and alpine oaks, respectively. Closely related species that produce fruiting bodies similar to *T. matsutake* exist in many parts of North Africa, North America, Central America, South America, as well as Europe and Asia (Redhead 1997). These are all collectively traded under the name matsutake. Among the global markets, Japan is by far the most important in terms of consumption. However, in the past century, because of deforestation and infestation by the pinewood nematode (*Bursaphelenchus xylophilus*), the host plant populations of *T. matsutake*, *Pinus densiflora*, declined rapidly in Japan (Gill *et al*. 2000; Wang & 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. In Europe, significant declines in ectomycorrhizal mushrooms, including species in the genus *Tricholoma* such as *T. matsutake*, have also been observed in many natural forest ecosystems (e.g. Arnolds 1991). 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 matsutake annually, mostly from Pacific North America, Korea and China. The commercial demands from Japan have subsequently placed serious pressure on natural populations of this species elsewhere, especially in southwestern China where up to a third of the Japanese imports come from. Indeed, the regions around Kunming, the provincial capital of Yunnan Province in southwestern China, once produced significant amounts of the matsutake mushroom over two decades ago but are now producing very little or none. As a result, understanding and conserving the natural populations of this mushroom have attracted significant attention in recent years from both government agencies and nongovernment conservation organizations.

Effective conservation and management strategies for any natural biological resources require a clear understanding of how the organisms reproduce in nature and how populations from different regions are related. For example, if sexual reproduction and recombination are common and important in a natural population, maintaining a set of mature mushrooms and their associated sexual spores will be important for its subsequent reproduction and survival in nature. In addition, if there is significant genetic differentiation among geographical populations, individual populations may need to be managed and conserved separately. At present, evidence for sexual reproduction in a local population of *T. matsutake* has been reported in Japan (Lian *et al*. 2006). However, the prevalence of sexual reproduction in other populations of *T. matsutake* is unknown. Furthermore, very little is known about the relationships between geographical populations, either in Japan or elsewhere. Only one study has examined the relationship between genetic distance and geographical distances in *T. matsutake* (Chapela & 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. A similar correlation was found in two species closely related to *T. matsutake, Tricholoma caligatum* and *Tricholoma magnivelare*, using five and six strains from diverse geographical locations, respectively (Chapela & Garbelotto 2004).

Since the 1980s, molecular markers have become increasingly important tools for studying a variety of biological properties and processes such as recombination and population structuring (Xu 2006a). Among the many types of markers that have been developed, single nucleotide polymorphisms (SNPs) are among the fastest developing category in biomedical and biological research. Though with its own caveats such as ascertainment bias (Morin *et al*. 2004), SNPs are the most frequently observed differences between DNA sequences obtained from different individuals or between alleles from within the same individual in diploid or higher ploidy organisms. In addition, SNPs have several properties such as a relatively low mutation rate and the ease of scoring and sharing data that make them highly desirable for a variety of biological analyses (Brumfield *et al*. 2003; Xu 2006a). Recently, we reported the identification of 178 SNPs for *T. matsutake* (Xu *et al*. 2007). Some of these SNPs were further distinguished using simple polymerase chain reaction (PCR) followed by digestions using specific restriction enzymes to generate restriction fragment length polymorphisms (RFLP). Here, using 14 PCR–RFLP markers developed previously based on the analysis of two strains, we genotyped 154 strains from 17 geographical regions from southwestern China. Our results indicate that sexual reproduction and recombination are prevalent in these populations and that there is limited but significant genetic differentiation among these geographical populations. However, we identified little genetic difference between samples obtained from the two different forest ecosystems, one dominated by pines and the other by alpine oaks and shrubs. The implications of these results in the taxonomy, conservation and sustainable utilization of *T. matsutake* are discussed.

Table 1 Populations of *Tricholoma matsutake* analysed in this study from southwestern China and their physical geographical information

Geographical population	Sample size (*N*)	Longitude (East)	Latitude (North)	Altitude (metres above sea level)
Baiyu	12	98.83	32.23	3040
Deqin	8	98.93	28.50	3400
Weixi	10	99.20	27.10	2320
Shangri-La	9	99.72	27.78	3280
Lijiang	9	100.25	26.86	2400
Lanping	11	99.20	26.4	2400
Longling	2	98.70	24.58	1540
Yongping	7	99.52	25.45	1620
Jianchuan	9	99.88	26.53	2195
Lincang	9	100.0	23.8	1780
Nanhua	9	101.27	25.22	1857
Lufeng	8	102.08	25.15	1600
Chuxiong	9	101.51	25.02	1775
Luquan	7	102.40	25.50	1679
Yimen	13	102.15	24.67	1580
Luliang	3	104.65	25.05	1840
Ailaoshan	19	102.20	23.15	2400
Total sample	154	98.70–104.65	23.15–32.23	1540–3400

Materials and methods

Mushroom samples

The isolates used in this study were collected in Yunnan and Sichuan Provinces in southwestern China. A total of 154 mushrooms were collected from 17 geographical locations. One location was in Baiyu County in western Sichuan Province that borders Tibet. The remaining 16 populations were from various regions in Yunnan Province, from Luliang in the east to Deqin in the northwest. The sample size and geographical coordinates for each population are presented in Table 1. The altitudes for all 17 populations are also given in Table 1. Our sampled regions spanned an area about 950 km from south to north and 650 km from east to west with an altitude span of almost 2000 m, from 1540 m above sea level in Longling to 3400 m in Deqin. All population samples were collected from mixed forests consisting of pines, alpine oak, and a variety of shrubs. The sampled forests in northwestern Yunnan (Deqin, Weixi, Shangri-La, and Lijiang) and Baiyu (Sichuan) were dominated by alpine oaks and shrubs. In contrast, those in other sampled regions in Yunnan (Lanping, Longling, Yongping, Jianchua, Lincang, Nanhua, Lufeng, Chuxiong, Luquan, Yimen, Luliang, and Ailaoshan) were dominated by pine trees. The sampled area contributes over 25% of the *Tricholoma matsutake* mushrooms harvested globally each year. Isolates of *T. matsutake* were identified based on their macro- and micromorphological characteristics and confirmed based on their sequences at the internal transcribed spacer (ITS) region of the ribosomal RNA gene cluster (see below).

While the detailed spatial maps of the fruiting bodies at the sampled geographical areas were not recorded, we have noted that none of the mushrooms we collected were clustered within 2 m of each other and that within each sampled population, the mushrooms were typically distributed from about 15 m of each other to as far as about 2 km from each other. The sampling distance was not subjectively imposed but was rather due to the rarity of fruiting of this species at any given time and the difficulty in finding the mushrooms in their native habitats. Indeed, within each of our sampled areas, all matsutake mushrooms we identified during our foray were collected. Because of their habit of producing fruiting bodies buried in the soil or the litter layer, matsutake mushrooms are difficult to find. As a result, it took us 6 years (2000–2006) of persistent effort to assemble this collection of 154 mushrooms from the 17 geographical regions. In several regions (Longling, Luliang, Luquan, and Yongping), aside from some initial successes, we failed to obtain any additional mushrooms despite repeated field trips. As reported by the local residents, these areas now produce very little or no matsutake mushrooms. While only two and three isolates were obtained from Longling and Luliang, they were the only ones we were able to obtain and were thus included here for analyses and comparisons.

DNA isolation

For each of the 154 strains, about 0.01 g tissues from the cap of the dried mushroom were ground into a fine powder using a blue tip in a 1.5-mL microcentrifuge tube. The remaining

steps essentially followed those for extracting DNA from live yeasts or from dried mushroom specimens (Xu *et al*. 1994, 2000), with slight modifications. The modifications were in the last steps. Briefly, after the DNA was washed with 70% ethanol and air-dried, they were re-suspended in 100 μL of Tris-EDTA (TE) buffer. The DNA was then cleaned using the commercial GeneClean III kit BIO101 (Qbiogene) following the supplier's instructions. The final DNA was suspended in 50 μL TE buffer and stored at –20 °C. By this method, 0.01 g of dried mushroom tissues yielded approximately 5 μg of genomic DNA.

Genotyping

Before the genotyping experiment using the restriction site polymorphic markers based on single nucleotide polymorphisms identified earlier in Xu *et al*. (2007), we sequenced a random set of strains at their nuclear ITS of the ribosomal RNA gene cluster using conserved primers ITS1 and ITS4 following a protocol described previously (Wang *et al*. 2007). The ITS sequencing was carried out both to confirm their species identification and to reveal potential sequence variation among isolates at this genomic region, especially strains from different forest types and geographical regions. In total, 34 isolates were analysed, with two isolates from each of the 17 geographical populations.

The genotypes of the isolates were obtained based on protocols described in Xu *et al*. (1999, 2007). A total of 14 PCR–RFLP markers located on seven random DNA fragments were screened for the entire 154 isolates. The 14 PCR–RFLP markers used here were three more than the original 11 presented in the Xu *et al*. (2007). The three additional markers were PCR fragment TmRC14 digested by the restriction enzyme *Hin*dIII, and PCR fragment TmRC18 digested by restriction enzymes *Hin*fI and *Ban*II, respectively. Information about obtaining both fragments was described in Xu *et al*. (2007). These PCR–RFLP markers were recently identified based on SNPs discovered through the analyses of a shotgun genomic library and the sequence comparisons among four alleles at 20 different loci in two mushrooms. For the genotyping, the DNA fragments that contained SNPs detectable by restriction enzyme digests were first amplified by PCR. Each PCR contained about 10 ng of DNA, 0.5 U *Taq* DNA polymerase, 1 μM each primer, and 200 μM of each of the four deoxyribonucleotide triphosphates in a total volume of 15 μL. The following PCR conditions were used for all amplifications: 4 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 50 °C, 45 s at 72 °C, and finally 7 min at 72 °C. After confirmation of the PCR products by agarose gel electrophoresis, the fragments were digested using the specific restriction enzyme. Typical restriction enzyme reactions consisted of 7-μL PCR product, 1 U restriction enzyme, 1.5 μL 10× reaction buffer, and H_2O to a total volume of 15 μL. Reactions were incubated 2–3 h at 37 °C or according to manufacturer's recommendations. PCR products and restriction digests were electrophoresed in 1.2% agarose in 1× TAE, stained with ethidium bromide, viewed by ultraviolet transillumination, and scored as codominant markers (Xu *et al*. 1999).

Data analysis

Our genetic analyses were performed for both within individual populations as well as among populations. In addition, the genetic distances among populations were compared to physical distances among geographical regions as well as their altitudinal differences to examine the potential relationships between genetic distances and geographical parameters. The specific analyses conducted here are briefly described below.

Analysis of genetic variation within populations. For the analysis of the within-population genetic variation, we calculated the genotype diversity and analysed the associations of alleles within the same locus as well as between loci for each of the 17 populations. Here, genotype diversity refers to the probability that any two individuals drawn randomly from the population will have a different multilocus genotype (Agapow & Burt 2001).

To examine whether there is evidence for recombination in individual geographical populations, we used two population genetic measures, Hardy–Weinberg equilibrium (HWE) test and the index of association. These two measures assessed the association of alleles within or between loci. The rationale for the inferences of recombination in these tests is that in a population with significant sexual reproduction and recombination, we should observe random associations between alleles at the same locus or between loci. This is because genes from different individuals are mixed every time sexual reproduction occurs, generating random associations between alleles at the same locus and between different loci (Xu 2006b). In diploid organisms with codominant genotype data, the HWE test has been used to examine the associations of alleles within each locus. Specifically, loci with genotype frequencies not significantly different from those expected under the assumption of random mating are determined to be in HWE. The predominance of loci in HWE indicates that recombination plays a significant role in the assayed population (Xu 2006b). The test for HWE was performed using the computer program GENALEX version 6 (Peakall & Smouse 2006) for each of the 14 loci in 15 of the 17 populations where sample sizes are sufficiently large. The exceptions were Luliang and Yongping (Table 2).

In the second test, we calculated the standard, most commonly used multilocus linkage disequilibrium called the index of association I_A using the computer program MULTILOCUS (Agapow & Burt 2001). In this test, the observed

Table 2 Patterns of genetic variation within geographical populations of *Tricholoma matsutake*

Geographical population	Percentage of loci that are polymorphic	Genotypic diversity	No. of loci in Hardy–Weinberg disequilibrium (Out of 14)	Index of Association
Baiyu	57.14	0.954	3	0.018
Deqin	50.00	0.893	3	0.318
Weixi	28.57	0.778	3	0.128
Shangri-La	35.71	0.861	2	0.133
Lijiang	35.71	0.583	3	0.182
Lanping	64.29	0.964	3	0.206
Longling	42.86	—†	—	—
Yongping	35.71	0.714	3	0.365
Jianchuan	42.86	0.972	2	0.039
Lincang	50.00	0.889	3	0.290
Nanhua	50.00	0.694	3	0.976*
Lufeng	42.86	0.893	3	0.014
Chuxiong	78.57	0.972	3	0.033
Luquan	42.86	0.952	3	0.331
Yimen	57.14	0.936	3	0.110
Luliang	35.71	—	—	—
Ailaoshan	57.14	0.895	3	0.836*
Total sample	100	0.966	2	0.193

*Statistically different from the null hypothesis of random association at $P < 0.05$; † not analysed because of small sample size.

data were compared against the null hypothesis that alleles (or genotypes) from different loci were randomly associating with each other. If no or little sexual reproduction occurred, there would be significant association between alleles at different loci due to clonal reproduction. In contrast, random associations between alleles at different loci suggest recombination (Xu 2006b). The formulae and inferences of statistical significance for this test can be found on the MULTILOCUS program homepage (Agapow & Burt 2001).

Analyses of genetic variation between populations. The genetic differences between samples were quantified using the phi-statistic through the analysis of molecular variance (AMOVA). Phi-statistics is a modified version of Wright's F_{ST} that refers to the relative contributions of between-population separation to the overall genetic variation in the whole sample. The greater the F_{ST} values are, the greater the differences between populations. The relative contributions of within-population genetic variation phiPT, between local populations within regions phiPR, and between regions phiRT were calculated using the computer program GENALEX (Peakall & Smouse 2006). Here, the dominant host tree species were used to define two regional-level populations, one dominated by alpine oaks and shrubs and the other by pines. Within the regional population dominated by oaks and shrubs are five populations from Baiyu, Deqin, Weixi, Shangri-La, and Lijiang. The other regional group includes the remaining 12 populations.

Tests for genetic isolation by geographical distance or altitudinal differences. The Mantel test was used to examine whether there was evidence for genetic isolation by geographical distance or by altitudes among our populations of T. matsutake. Because detailed sporocarp–sporocarp distances were not collected for most of the isolates within each geographical area, the potential genetic isolation by geographical distance within individual populations could not be assessed. Instead, our analyses focused on the relationships between populations. To this end, we conducted two separate Mantel tests. In the first Mantel test, the pairwise Nei's population genetic distances were calculated based on gene frequency differences between populations and were then compared to geographical distances between sampled populations. Our second Mantel test compared Nei's genetic distances with altitudinal differences between populations. Both Mantel tests were conducted using the program GENALEX (Peakall & Smouse 2006).

Results

Our ITS sequence results identified that all 34 sequenced isolates had ITS sequences identical to each other and to the GenBank ITS sequence of the typical *Tricholoma matsutake* isolates from Japan, Korea, and China. In contrast, their ITS sequences were distinctly different from those in other matsutake mushrooms such as *Tricholoma bakamatsutake* and *Tricholoma magnivelare*. Unfortunately, no sequence

information, including ITS sequence, is available in GenBank for strains of *Tricholoma zangii*, including the type specimen, for comparison. Despite the absence of such information, the ITS results we obtained here are consistent with the hypothesis that the mushrooms analysed here belonged to one species, *T. matsutake*, and not its allied species. In addition, we saw no difference in ITS sequences between those isolated from pine-dominated forests and those from alpine oak-dominated forests. The GenBank Accession numbers for the 34 ITS sequences are EU294269–EU294302. Because the ITS sequences for the 34 *T. matsutake* isolates from the same or different regions in southwestern China were all identical, we did not conduct any further population genetic analysis of the ITS sequence data. Below, we present the analyses of the genotype information based on SNP markers.

Genetic variation within geographical populations

Our analyses identified abundant genetic variations within each of the 17 geographical populations of *T. matsutake* from southwestern China. The analysed loci showed a high discriminating power among individuals. Based on results from randomizations, any eight of the loci analysed here were sufficient to achieve a very high discriminating power (Fig. 1), indicating a level of saturation for identifying unique genotypes using the 14 SNP markers. The overall genotypic diversity for the whole sample of 154 individuals was 0.966, suggesting that over 96% of the time, two randomly drawn individuals from the total population will have genotypes different in at least one of the assayed loci. There is, however, a range of variation in genotypic diversity among the 17 geographical populations, from a low of 0.583 in Lijiang to a high of 0.972 in both Jianchuan and Chuxiong (Table 2).

The overall results from the HWE tests suggest that most loci in most populations were in Hardy–Weinberg equilibrium, a result consistent with the hypothesis that recombination plays an important role in the natural populations of this species. Interestingly, most populations have two to three loci in Hardy–Weinberg disequilibrium (Table 2). A close examination of the genotype data indicated that two of the 14 polymorphic restriction sites consistently showed excess heterozygosity in all of the populations. These two sites were located on the same DNA fragment, RC14 (the raw genotype data are not shown here but see Table 2 in Xu *et al*. 2007 for the representative excess heterozygosity).

Results from the index of association analyses similarly suggested widespread random associations among alleles at different loci in these populations. Specifically, only two of the geographical populations showed any evidence of significant allelic associations among loci, one population from Nanhua and the other from Ailaoshan. When all the 154 strains were analysed together, we saw no evidence of significant allelic associations among loci (Table 2).

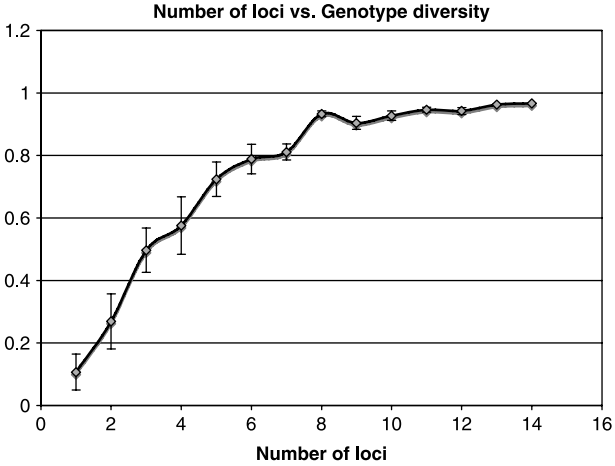

Fig. 1 Permutation analysis showing the relationship between the number of assayed loci and genotype diversity in the total population of *Tricholoma matsutake* from southwestern China. The *X*-axis shows the number of randomly analysed loci and the *Y*-axis shows the genotype diversity. Each data point shows the mean and standard deviation of genotype diversity from 100 permutations. The maximum genotype diversity is 1 when all strains in the population have different multilocus genotypes. The minimum genotype diversity is 0 when all analysed strains have the same multilocus genotype. Note the tapering off of genotypic diversity with eight random loci analysed.

Genetic variation among geographical populations

Table 3 shows the F_{ST} values between pairs of geographical populations. Overall, the mean F_{ST} value was about 0.10, indicating that about 10% of the gene diversity was due to geographical separations between pairs of populations. The lowest F_{ST} value (0.007) was found between Weixi and Lijiang while the highest (0.232) was between Weixi and Luliang (Table 3). The AMOVA results suggested that all three levels contributed significantly to the overall genetic variation, with the regional level contributing the least (Table 4). Specifically, the host tree species (i.e. between regions) contributed 9% of the total genetic variance. The next level, between populations within regions, contributed 34% of the total genetic variance, while the remaining 57% genetic variance came from within individual populations (Table 4). Permutation analyses indicated that the AMOVA results were consistent with significant genetic differentiations among the 17 analysed populations ($P < 0.01$ for phiRT, phiPR and phiPT in the AMOVA tests).

The relationship between genetic distance and geographical parameters

The results from the Mantel tests are shown in Fig. 2. The test showed a significant positive correlation between genetic distance and geographical distance among the analysed

Table 3 Pairwise F_{ST} values between geographical populations of *Tricholoma matsutake* from southwestern China

	Deqin	Weixi	Shangri-La	Lijiang	Lanping	Longling	Yongping	Jianchuan	Lincang	Nanhua	Lufeng	Chuxiong	Luquan	Yimen	Luliang	Ailaoshan
Weixi	0.093															
Shangri-La	0.065	0.019														
Lijiang	0.088	0.007	0.023													
Lanping	0.032	0.070	0.062	0.064												
Longling	0.041	0.080	0.062	0.072	0.023											
Yongping	0.070	0.016	0.005	0.018	0.053	0.049										
Jianchuan	0.115	0.143	0.129	0.123	0.064	0.107	0.121									
Lincang	0.079	0.093	0.100	0.080	0.031	0.040	0.076	0.111								
Nanhua	0.057	0.022	0.030	0.022	0.041	0.051	0.028	0.097	0.059							
Lufen	0.033	0.105	0.080	0.099	0.037	0.020	0.076	0.152	0.071	0.066						
Chuxiong	0.074	0.096	0.102	0.086	0.038	0.065	0.086	0.097	0.027	0.071	0.089					
Luquan	0.137	0.170	0.161	0.143	0.068	0.108	0.143	0.015	0.094	0.117	0.164	0.083				
Yimeng	0.050	0.093	0.087	0.081	0.013	0.040	0.073	0.047	0.037	0.059	0.070	0.036	0.045			
Luliang	0.188	0.232	0.230	0.195	0.097	0.144	0.202	0.070	0.101	0.164	0.208	0.083	0.026	0.072		
Ailaoshan	0.115	0.111	0.113	0.095	0.089	0.121	0.104	0.109	0.107	0.079	0.164	0.069	0.116	0.079	0.147	
Baiyu	0.048	0.099	0.085	0.089	0.057	0.093	0.091	0.085	0.102	0.060	0.107	0.075	0.114	0.057	0.165	0.069

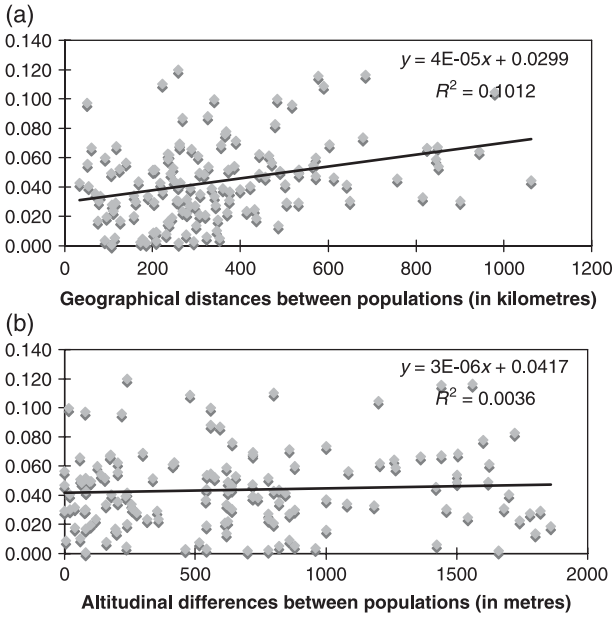

Fig. 2 Results from two Mantel tests between genetic differences and geographical distances among populations. (a) A Mantel test between Nei's genetic distance and the two-dimensional geographical distances (based on longitudinal and latitudinal coordinates) among populations. (b) A Mantel test between Nei's genetic distance and altitudinal differences between populations. In both 2a and 2b, the *X*-axis represents the geographical distance parameter and the *Y*-axis represents Nei's genetic distances between populations.

populations (Fig. 2a; $P = 0.028$), with a correlation coefficient of 0.318. However, we found no correlation between altitudinal differences and population genetic distances (Fig. 2b; $P = 0.272$).

Discussion

In this study, we analysed a large number of geographical populations of the ectomycorrhizal mushroom *Tricholoma matsutake* from southwestern China. Our results identified significant genetic variation within and between populations. There was evidence for recombination within each of the analysed populations. Overall, these populations showed relatively low but significant genetic differentiation. In addition, the amount of differentiation varies between populations, with the level of differentiation correlated to some extent to geographical distances separating the populations, consistent with results from a previous study using different samples (Chapela & Garbelotto 2004). Our analyses identified no correlation between genetic distance and altitudinal differences among populations. In addition, the habitat differences as represented by the dominant host trees contributed about 9% to the total genetic variation, less than a third of that between populations within habitats (34%) and a sixth of that within individual populations (57%) (Table 4).

Table 4 Summary results of the analysis of molecular variance (AMOVA) within and among populations of *Tricholoma matsutake* from southwestern China

Source	d.f.	SS	MS	Estimated variance	Percentage	Stat	Value	*P*
Between regions	1	20.661	20.661	0.184	9	PhiRT	0.086	0.010
Among populations within regions	15	114.446	7.630	0.719	34	PhiPR	0.368	0.010
Within populations	137	169.399	1.236	1.236	57	PhiPT	0.422	0.010
Total	153	304.506	29.528	2.139				

d.f., degree of freedom; SS, sum of squared observations; MS, mean of squared observations; PhiRT, proportion of the total genetic variance that are between regions; PhiPR, proportion of the total genetic variance that are among populations within a region; PhiPT, proportion of the total genetic variance that are among individuals within a population

The abundant genetic variations found in this study and in our recent study (Xu *et al*. 2007) among strains and populations of *T. matsutake* from southwestern China form a stark contrast to previous results obtained using either the repetitive element MarY1 for fingerprinting (Murata *et al*. 2005) or sequence polymorphism at the ITS regions and the intergenic spacer (IGS) region of the ribosomal RNA gene cluster (Sha *et al*. 2007). In the study by Sha *et al*. (2007), virtually no polymorphism was found among 56 fruiting bodies collected from 13 counties in Yunnan when analysed using a specific PCR primer that had been shown to be highly polymorphic for strains from Japan. Our samples here included all 56 strains from the 13 locations in the study by Sha *et al*. (2007). Though we have been unable to obtain samples from Japan for comparison using our genotyping method, the differences in the MarY1 fingerprinting patterns between the Chinese and the Japanese samples seemed to suggest that there might be significant genetic differentiation between the Japanese populations of *T. matsutake* and those from southwestern China.

Our within-population genetic analyses suggest that sexual reproduction and recombination are widespread among the geographical populations from southwestern China (Table 2). This conclusion is similar to what has been found in one Japanese population of *T. matsutake* where Hardy–Weinberg equilibrium was observed for four microsatellite loci in a local population (Lian *et al*. 2006). However, in our study, two polymorphic nucleotide sites on the same DNA fragment showed consistent excess of heterozygosity across all populations analysed here. While the detailed mechanism for this excess of heterozygosity is unknown, there may be several possibilities. One possibility might be due to ascertainment bias where the analysed locus was highly polymorphic but might not be representative of the rest of the genome. Ascertainment biases have been found in many SNP applications in a variety of species (Morin *et al*. 2004). In fungi, DNA fragments located within the mating-type locus or close to the mating-type locus often exhibit excess heterozygosities in natural populations. It is possible that this locus was located within or adjacent to the mating-type locus in *T. matsutake*. Indeed, in heterothallic fungi, heterozygosity at the mating type locus is required for fruiting body formation (Raper 1966). Unfortunately, little is known at present about the mating system in *T. matsutake*, including the number of segregating loci that control mating compatibility and the number of functional alleles at each locus. The inferred recombination from our data and those of Lian *et al*. (2006) and of Xu *et al*. (2007) do suggest, however, that *T. matsutake* is likely a heterothallic species. Other possibilities for excess heterozygosity include (i) heterozygous advantage due to complementary gene actions, and (ii) genomic rearrangements that inhibit recombination and maintain heterozygosity (Xu 1995).

In several of our populations, the analysed sample sizes were very small. This was especially true for two populations, Longling and Luliang, where only two and three isolates, respectively, were obtained and analysed in this study. The small sample sizes and the relatively long distances between specimens within each of the populations make our samples of limited use to infer the sizes of genetic individuals in natural populations of this species in southwestern China. A previous study in seven different sites in Japan identified that the average size of *T. matsutake* genets was about 2 m in the longer dimension, with the largest about 11.5 m and the rest ranged from 0 to 5.0 m (Lian *et al*. 2006). Interestingly, some mushrooms located very close to each other and thought to belong to the same 'shiro' ('genet' in Japanese) were found to have different genotypes at the assayed microsatellite loci (Lian *et al*. 2006). If the genet sizes of *T. matsutake* in southwestern China were similar to those in Japan, the vast majority of the strains analysed here would represent different genets or 'shiros'. Therefore, we believe it unlikely that the low genotype diversity observed in some populations such as Lijiang was due to repeated sampling of mushrooms from unusually large genets. The small genet sizes identified in Japan in combination with the small sample sizes in many of the populations convinced us not to do analysis using clone-corrected samples. The

establishment of long-term experimental sites would be needed to allow us to critically examine the size of genetic individuals and the dynamics of microscale genetic variation in natural populations of this species in various regions in southwestern China.

The F_{ST} values observed between geographical populations of *T. matsutake* are similar to those reported in several basidiomycete species (James *et al*. 1999; Xu *et al*. 2005). For example, between regional populations of the button mushroom, *Agaricus bisporus*, the F_{ST} values were found ranging between 0.019 and 0.076 (Xu *et al*. 1997). In the oyster mushrooms *Pleurotus ferulae* and *P. eryngii*, 19 and 6 regional populations from Italy showed F_{ST} values of 0.45 and 0.10, respectively, for these two species (Urbanelli *et al*. 2003). The intercontinental populations of the model basidiomycete *Schizophyllum commune* were significantly differentiated when examined using isozyme markers, with an F_{ST} value of 0.214 (James *et al*. 1999). In *T. matsutake*, Chapela & Garbelotto (2004) identified that among the seven strains they analysed, they found a positive correlation between their pairwise genetic dissimilarity and geographical distances. Those seven strains were far apart from each other (up to over thousands of kilometres) and included three from China (one northeastern China and two from southwestern China) and one each from France, Japan, Korea, and Morocco. 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.

The statistically significant genetic differentiation between populations from forests dominated by different tree species seems to suggest that these populations are genetically distinct from each other. However, our analyses indicated that the level of differentiation between the two forest types was low, less than one-third of the genetic differences between populations from within the same forest types and less than one-sixth of the genetic variation contributed from within local individual populations (Table 4). The lack of sequence variation at the ITS region between *T. matsutake* and *T. zangii*, and our population genetic analyses presented here suggest that it has to be addressed by further detailed studies whether the taxonomic separation of *T. zangii* [syn. *T. quercicola*] (Zang 1990; Cao *et al*. 2003) and *T. matsutake* is justified.

While much remained unknown, many factors have been suggested to influence the productions of natural ectomycorrhizal mushrooms, including those of *T. matsutake*. These factors include both biotic factors such as the type and age of vegetation in the forests and abiotic factors such as nutrient levels, pH, and air quality (Arnolds 1991). For example, in Europe, forests with pine trees more than 40 years old have shown to exhibit reduced ectomycorrhizal mushroom production than those with younger trees. Elevated levels of organic debris on the forest floor and nitrogen levels in the soil have also been observed to contribute to decreased production of *T. matsutake*. Aside from better management decisions based on these observations, we believe our population genetic study can also contribute to improved management strategy on sustainable resource utilizations of *T. matsutake* in southwestern China. Specifically, the observation that sexual recombination plays a significant role in all the study populations in southwestern China suggests that sexual spores are important propagules for the reproduction of this species in nature. 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 sexual reproduction. At present, in order to satisfy consumer demands in Japan, virtually all *T. matsutake* mushrooms are picked prematurely before the veils of the mushrooms are ruptured to release spores. Leaving a few mushrooms at each site to mature and sporulate, in combination with careful harvesting techniques that disturbs little of the underground mycorrhizae, should help populations to sustain. In addition, the low but significant genetic differentiations among geographical populations observed here suggest that while long-distance gene flow is present, such a gene flow might not be strong enough to counter local genetic differentiation between certain populations. As a result, whenever possible, each site should leave a few mushrooms to mature and sporulate to ensure its continued reproduction.

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