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Forest age correlates with fine-scale spatial structure of Matsutake mycorrhizas

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ABSTRACT

Examining the fine-scale spatial structure of fungal populations can tell us much about how individual species reproduce and disperse throughout natural landscapes. Here we study the fine-scale genetic structure of *Tricholoma matsutake*, a prized edible and medicinal mushroom, by systematic sampling of mycorrhizas within fairy rings in 50-y-old and old-growth forests in two villages. Using single nucleotide polymorphism DNA markers we show that mycorrhizas in both forest age classes in both villages showed high levels of genotypic diversity, consistent with a reproductive life history predominated by outcrossing via basidiospore dispersal. Both the percentage of polymorphic loci within fairy rings, as well as genotype diversity were higher in old-growth compared to 50-y-old forests. Fifty-year-old forests showed significant spatial autocorrelation between pairs of mycorrhizas up to 42 m, and a pattern consistent isolation-by-distance structure. Spatial patterns in old-growth forests were random. Furthermore, AMOVA analysis indicates that 11 % of molecular variance in 50-y-old forests is partitioned between villages, whereas no significant variance is partitioned between villages in old-growth forests. We conclude that populations of *T. matsutake* in 50-y-old forests are the result of a founder effect maintained by local inoculation sources. This pattern attenuates as forests age and accumulate inocula from more distance sources. We speculate on how genetic mosaicism within *T. matsutake* fairy rings may structure populations within a chronosequence. Finally, we discuss how population spatial dynamics and dispersal strategy in *T. matsutake* contrast with other ectomycorrhizal species.

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Introduction

Ectomycorrhizas are cosmopolitan in temperate forests where they play an essential role in mineral uptake, nutrient cycling, shaping plant communities, and providing food and economic livelihoods to humans (Francis & Read 1994; Koide & Dickie 2002; Yun & Hall 2004). There have been many studies characterizing the alpha diversity of ectomycorrhizal communities

in recent years (Horton & Bruns 2001) (Dighton *et al.* 2005), however relatively few studies have examined ectomycorrhizal fungi (EMF) at the population or individual scale.

Examining the fine-scale spatial structure of EMF populations can tell us much about how individual species reproduce, persist and disperse throughout natural landscapes. A review by Deacon and Fleming (1992) of successional guilds in EMF suggests that species may be classified as “early

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stage”: characterized by low sugar requirements, small and ephemeral sporocarps, with easily germinated spores; or as “late stage”: characterized by high sugar requirements, large and persistent sporocarps with spores that are difficult to germinate. According to this paradigm, early stage species would spread predominantly via spore dispersal in young and disturbed habitats, whereas late stage species would spread predominantly via long-lived hyphal growth in older and less disturbed habitats. Because the successful establishment of a genetic individual is contingent upon available space, early stage species, which extend their range via spores, have a clear advantage when competition for space is minimized and dispersal ability is at a premium. Conversely, for late stage species where both inter- and intra-specific competition plays a role, vegetative hyphal growth may confer advantages (Dahlberg & Stenlid 1995).

Plot-level studies of spatial population structure of EMF have demonstrated an apparent continuum from large genetic clones which are vegetatively propagated in species such as *Suillus bovinus* (Dahlberg & Stenlid 1990, 1994) and *Xerocomus* spp. (Fiore-Donno & Martin 2001) to much smaller genetic clones or individuals which likely propagate via basidiospore as has been found in *Hebeloma cylindrosporum* (Gryta et al. 1997; Guidot et al. 1999), *Laccaria amythestina* (Fiore-Donno & Martin 2001; Gherbi et al. 1999), *Cantharellus* spp. (Dunham et al. 2003), *Lactarius xanthogalactus*, *Russula cremoricolor*, and *Amanita francheti* (Redecker et al. 2001). *Russula brevipes* has been shown to display intermediate characteristics, with many genotypes represented by single individuals or small (<3 m) clusters and others represented by mushrooms located 18 m from each other (Bergemann & Miller 2002).

A handful of studies have measured whether habitat, site, or other environmental factors affect intra-specific genet size. A number of these studies have illustrated cases where genet size of a species positively correlates with forest age (i.e. Dahlberg, 1997; Dahlberg & Stenlid 1990, 1995). Similarly, Guidot et al. (2002) found larger genets of *Hebeloma cylindrosporum* in relatively undisturbed “dune” sites (2.05 ± 0.72 m) compared to disturbed “forest” sites (0.23 ± 0.08 m). There have been demonstrated exceptions to this trend however: a study of two species of *Cantharellus* replicated in old-growth, recently thinned old-growth and 40–60-y-old Douglas fir forest plots showed mean genet sizes for the two species of 3.2 ± 3.6 m and 1.5 ± 1.7 m with no evidence for statistical differences between habitats (Dunham et al. 2003). The authors of that study caution that the presence of unresolved genotypes, null alleles and fragmentation of larger genets by novel infections may introduce error into these measurements. Fiore-Donno & Martin (2001) and Gherbi et al. (1999) also found small *Laccaria amythestina* genets in mature forests, suggesting that, regardless of habitat, vegetative growth is rare in that species.

Because sporocarp material is relatively easy to identify, preserve, and extract DNA from, it has served as the individual sampling unit in nearly all fine-scale spatial studies of ectomycorrhizal species forming above-ground fruit bodies. The advantages that sampling sporocarps confer are countered by the fact that mushroom presence is often ephemeral and unpredictable, requiring repeated sampling over the span of an entire season at the least. A further drawback may be

that sporocarp genotypic diversity and spatial structure differs from that of below-ground structures. Some previous studies have shown contrasting patterns of correspondence between above-ground mushrooms and below-ground mycorrhiza genotypes. Guidot et al. 2001.’s study of the pioneer EM mushroom *Hebeloma cylindrosporum* (2001) found that all mycorrhiza genotypes were located within 20 cm from a corresponding fruit body, and that mushroom disappearance at a given location coincided with the disappearance of the corresponding mycorrhiza genotype within 1 y. Conversely, Dahlberg et al. (1997) observed high densities of *Cortinarius* spp. mycorrhizas in areas where no fruit bodies were found. Sampling mycorrhizas themselves provides challenges both in detection and identification, but may improve fine-scale sampling of populations by providing a more complete spatial and genotypic characterization of ectomycorrhizal populations (Kretzer et al., 2005).

We chose to study the fine-spatial-scale genetic structure of *Tricholoma matsutake*, a prized edible and medicinal mushroom. This mushroom has been revered in Japan for centuries for its distinguished flavor, medicinal properties and iconic significance (Hosford et al. 1997; Redhead 1997). *Matsutake* is among the costliest mushrooms on earth: in the 1990s, at the start of the mushroom season, wholesale prices in Japan reached as high as US\$1275/kg (Yun et al. 1997). In rural Zhongdian county, China, where this study took place, it is estimated that 80 % of farmers participate in the *Matsutake* trade (Yun & Hall 2004).

Tricholoma matsutake conforms to many of the postulates of late stage mycorrhizal species: its spores are difficult to germinate under laboratory conditions (Ohta 1986), its sporocarps are large and long-lived, and it produces persistent, dense mycelial mats known as fairy rings or *shiro* (Hosford et al. 1997; Murata et al. 2005). Two recent studies have shown that genotypic diversity exists at small spatial scales within these fairy rings. A study by Lian et al. (2006) of *T. matsutake shiro* in Japan used four polymorphic microsatellite markers to genotype mushrooms forming fairy rings in an 85-y-old Pine forest and the mushroom’s correspondence with mycorrhizas located directly below and adjacent. Their study identified a corresponding mycorrhiza genotype directly beneath each mushroom sampled, as well as two additional genotypes located 30 cm and 1 m away. No *Matsutake* mycorrhizas were found outside of the *shiro*. This is consistent with Murata et al.’s (2005) study which used an inter-retrotransposon amplified polymorphism marker to characterize a high degree of genetic mosaicism within *T. matsutake* rhizospheres and mushrooms collected over multiple years.

These spatial patterns contrast with those found in studies of fairy rings of saprobic fungi, which have been shown to consist of single genotypes (Abesha et al. 2003; Dowson et al. 1989; Mallett & Harrison 1988). While fairy ring formation *per se* is rare in EMF, a number of studies have shown relatively large aggregations of single non-overlapping genotypes (Bonello et al. 1998; Dahlberg & Stenlid 1990, 1994; Sawyer et al. 1999).

We hypothesized that *Matsutake*, *vis a vis* genetic mosaicism in fairy rings, may contradict the paradigm that late stage fungi are characterized by large persistent genets. A second aim of our study was to test whether *Matsutake* populations are predominantly characterized by spore or mycelia

dispersal. Last, we tested whether forest age affects *T. matsutake* genetic spatial structure via spatial autocorrelation and isolation-by-distance patterns.

Materials and methods

Field site

Study sites were located in the forests of the villages of Jidi and Wengshue in Shangri-la County, Yunnan, China, 28.6° N, 99.5° E (Fig 1). Villages were selected for known high Matsutake mushroom productivity and for known forest histories. Sampling sites are located in mixed conifer deciduous forests dominated by *Pinus* spp. and *Quercus* spp. overstory. Both villages have forests (50 y old) which had been collectively managed for mixed use (including timber and non-timber product harvest) prior to clear-cutting in the 1960s concurrent with China's "Great Leap Forward". Both villages also had forests where no logging had ever been known to have occurred: due to location on a mountain associated with resident deities in the case of Wengshue, and due to location on a relatively distant slope in the case of Jidi. Historically and at present,

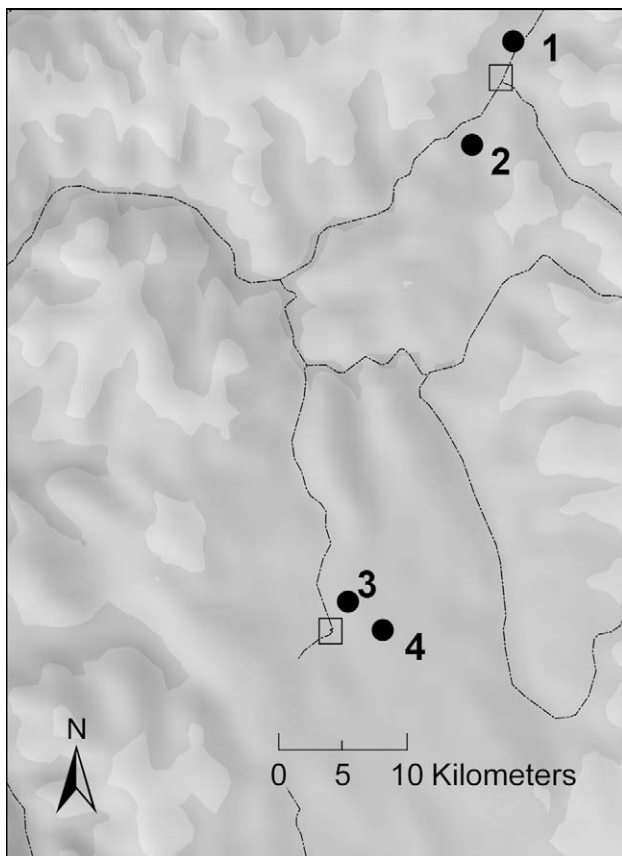


Fig 1 – Map of villages and sampling locations. Wengshue 50-y-old (1), Wengshue old-growth (2), Jidi 50-y-old (3), and Jidi old-growth forest samplings sites are designated with closed circles. Locations of villages are depicted with open squares. Broken lines are rivers. Background shading is 1000 m contour intervals with the darkest shades at lowest elevations.

mushroom collection is permitted in these forests, and both are located sufficiently near to roads that harvest is intensive in all locations surveyed in this study. A subset of large *Pinus* trees in both age classes was analyzed with an increment borer, and average tree age was 45 ± 7.9 y and 86 ± 12.3 y in 50-y-old and old-growth forests respectively.

Vegetation and environmental characterization

Within each forest age class, *shiro* locations (see below) were used as the center point for a point-center-quarter, plotless sampling protocol as in Mueller-Dombois & Ellenberg (1974). The diameter of trees larger than 5 cm diameter at breast height (DBH) were measured and distance from the center point was used to calculate tree density. Soil pH was measured from approximately 1 kg bulk mineral soil collected from three replicates in each forest age class in each village. Results of these analyses are summarized in Table 1.

Mycorrhiza sampling

Microsites known to have produced *T. matsutake* mushrooms within the 2 y prior to the study were targeted for soil sampling. Soil cores (3 cm diameter) were extracted to a depth of 10 cm at three points along a transect at 0.5, 1, and 1.5 m from a central point. Cores were taken along three such transects within each *shiro*, totaling nine cores (Fig 2E). Field confirmation of *T. matsutake* presence was possible because of the distinct hydrophobic mat-forming mycelia and olfactory detection of methyl cinnamate, a volatile compound which gives *T. matsutake* a distinctive smell (Cho et al. 2008; Wood & Lefevre 2007; Yajima et al. 1981). Fifteen *shiro* were sampled in each of the four plots between June and August 2005. Soil cores were kept at 4 °C until further processed within 2 weeks.

Soil cores were gently washed over a screen and *T. matsutake* mycorrhizas were putatively identified under a dissecting microscope. During preliminary studies, *T. matsutake* mycorrhizas were isolated directly from the base of sporocarps, facilitating their later identification from soil samples. *T. matsutake* mycorrhizas on pine roots tend towards dense clusters. The mycorrhizas form a distinctive blackening hyphal mantle which progresses towards the root apex. Emanating hyphae can be abundant on colonized root tips in which the fungal mantle has not completely colonized the root radical. Detailed descriptions (Ogawa 1975a) and photographs of *T. matsutake* mycorrhizas on Pine roots (Gill et al. 2000) have previously been published. Six *T. matsutake* mycorrhizas were haphazardly selected from each soil core for genetic analysis. Mycorrhizas were dried in open 0.5-ml Eppendorf tubes, partially submerged in silica gel in vacuum chambers.

DNA isolation

Dried mycorrhizas were incubated in 10 μ l of extraction solution from the Extract-N-Amp Plant PCR Kit (Sigma Aldrich, St. Louis, MO) for 10 min at 65 °C followed by 15 min at 95 °C. 10 μ l of dilution solution was added and vortexed briefly. Genomic DNA extracts were diluted twofold in 0.1 \times TE buffer (10 mM Tris pH 8.0, 1 mM EDTA-Na) and stored at -20 °C for subsequent PCR.

Table 1 – Summary of characteristics for sampling locations

	Trees/Ha	Soil pH	Pinus DBH (cm)	Average elevation (m)	Approximate distance from road (km)
Jidi OG	795.73	5.22 ± 0.38	22.98 ± 10.19	3500	3.0
Weng OG	1259.57	6.19 ± 0.19	17.33 ± 7.62	3542	0.7
Jidi 50	906.33	5.69 ± 0.68	17.27 ± 8.20	3505	1.3
Weng 50	1064.48	5.81 ± 0.2	16.05 ± 6.56	3336	0.3

Species identification for 10 isolates was verified by sequencing the internal transcriber spacer (ITS) portion of the nuclear ribosomal operon, using the primer pair ITS1f and ITS4 (White et al. 1990) and by comparing the resulting DNA sequences with sequences deposited in GenBank through the BLAST algorithm on the NCBI GenBank website (<http://www.ncbi.nlm.nih.gov>).

Mycorrhiza genotyping

Several different molecular markers were assayed for variation among samples from this study. Ten microsatellite markers described by Lian et al. (2003) for a Japanese population of *T. matsutake* were tested on a large subset of isolates. Of those loci, only one, TM16, showed variation in a mono-nucleotide repeat motif. These loci were not used for subsequent analyses. A microsatellite enrichment library was constructed in the Glenn Laboratory (University of Georgia) as in the methods of Glenn and Schable (2005). Thirty-five primer pairs were used to amplify genomic DNA from eight mushroom isolates from eight populations within roughly 150 km from this study's sampling locations. None of these microsatellites showed polymorphism, and were not used in subsequent analyses.

Five variable single nucleotide polymorphism (SNP) markers were designed either from the shotgun clone library

sequence published by Xu et al. (2007) or from a clone library sequence contig of the microsatellite enrichment described above. TmRC 4 is a 455-bp fragment (characterization and primers are in Xu et al. 2007), while TmSNP 8 is a 346-bp fragment located adjacent to a microsatellite sequence identified in this study. TmSNP 8 was amplified using the primer pair TmSNP 8 forward: TGG GTT GGG TTG TAT CCA TT, and TmSNP 8 reverse: ATT CTC TGC TCG GAC TCT GC. The two fragments were amplified with conditions as follows: 1 µl DNA was added to a PCR reaction containing 1 µM each primer, 1.5 mM MgCl₂, 200 µM of each dNTP and 0.25 units of Taq DNA polymerase to a final volume of 10 µl. PCR reactions were initially denatured at 95 °C for 30 s followed by 25 cycles of 10 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C and a final extension of 5 min at 72 °C.

One microliter of PCR product was added to a second PCR reaction containing 1 µM of each nested primer (TmRC4F_nested: TCC ACC ACC TCT CTG GGT AG and TmRC4R_nested: AAT TGA TGC CAT GGG GTA TG; and TmSNP 8F_nested: TGG GTT GGG TTG TAT CCA TTT AT and TmSNP 8R_nested: AAA GTG CTC AGA CTA CCC CTC TT), 1.5 mM MgCl₂ and 200 µM of each dNTP. 0.75 units of Taq DNA polymerase was added to a total volume of 30 µl for TMRC 4, and 0.25 units of Taq was added to a final volume of 10 µl for TmSNP 8. PCR reactions were initially denatured at 95 °C for 30 s followed by 35 cycles of 10 s at 95 °C, 30 s at 55 °C, 30 s

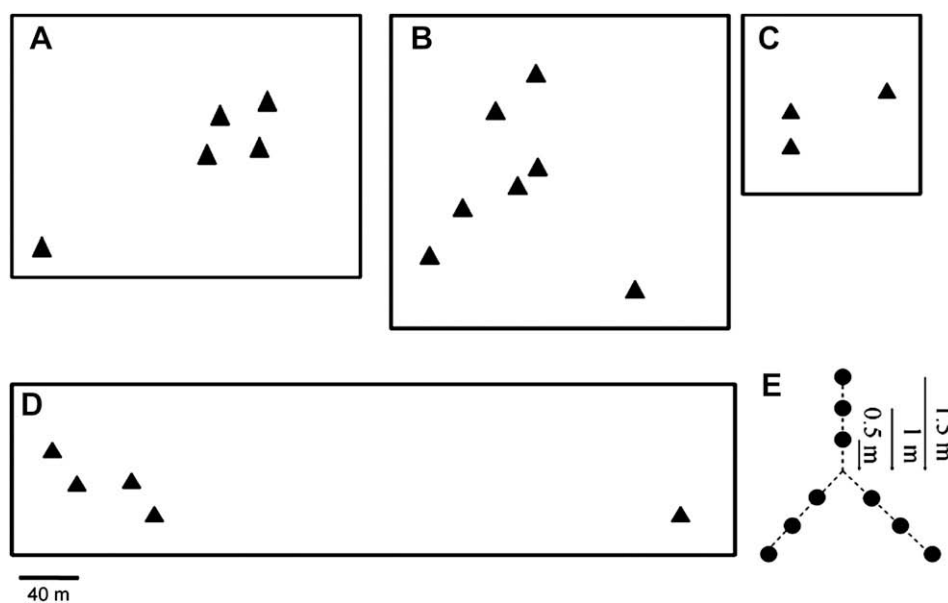


Fig 2 – Plots of *T. matsutake* shiro genotyped. Plots represent Jidi old-growth (A); Wengshue 50-y-old (B); Jidi 50-y-old (C); Wengshue old-growth (D). Each triangle represents a shiro location where nine soil cores were sampled and at least one mycorrhiza was recovered and genotyped. Single cores were extracted at 0.5 m intervals (closed circles, E).

at 72 °C and a final extension of 5 min at 72 °C. Restriction enzymes complementary to a single SNP allele and nowhere else on the DNA fragment were selected for a PCR-RFLP genotyping assay. TMRC 4 was digested with *Acil*, *AleI*, *EcoRI* and *SphI* restriction enzymes, and TmSNP 8 was digested with *BstBI* restriction enzyme (New England Biolabs, Ipswich, MA). Eight microliters of PCR product were added to two units of restriction enzyme and 2 μ l of 10 \times reaction buffer, and reactions were incubated for 6 h at 37 °C.

Reactions were electrophoresed in 2% agarose in 1 \times TAE and scored as codominant markers. For every individual, each allele for both haplotypes was designated with “1” (undigested) or “2” (digested) in a spreadsheet. Homozygotes were distinguished as having either a single electrophoretic band (scored 1/1) or two bands (scored 2/2), and heterozygotes were distinguished as having three bands (scored 1/2).

Taxon specificity was assumed as primers failed to cross-amplify DNA isolated from sporocarps of *T. bakamatsutake*, the most closely related sympatric EMF known in the study sites. Conversely, due to complete PCR amplification efficiency of genomic DNA extracted from *T. matsutake* sporocarps in a parallel study (see below), we were confident that null alleles were avoided.

Data analysis

Multilocus genotypes were compiled (see summary in Table 2). Identical multilocus genotypes originating from the same *shiro* were removed for all analyses excepting AMOVA. Observed genotype diversity was calculated both including and excluding identical genotypes from within the same *shiro*.

Because our samples were not independent or randomly surveyed, they were not useful for inferring marker independence, or Hardy Weinberg equilibrium (HWE). Therefore, we relied on additional data to assess the extent of linkage disequilibrium and random mating in the population as a whole. In a separate analysis of 209 sporocarps from eight populations in a wider geographic range (approximately 400 km²) no populations showed significant I_A . The SNP located at restriction site *EcoRI* on fragment TmRC4 was found to be in linkage disequilibrium with *AleI* and *Acil* in five of eight populations. In this same study an average of 0.87 loci (of six) were found to have an excess of homozygotes within each population. Other studies of *T. matsutake* in NW Yunnan have found little evidence of population structure at comparable spatial scales within a watershed (Amend unpublished data; Xu et al. 2008).

For this reason, in this study we deemed it appropriate to consider all mycorrhizas as a single population and to pool all isolates for measuring pair-wise linkage disequilibrium. This analysis was conducted using the program Genepop on the Web (Rousset & Raymond 1995) and no significant linkage between any loci was found (data not shown). Expected and observed heterozygosity for each forest age class were likewise calculated using Genepop on the Web (Table 2). Here we intend this statistic as a qualitative measure of diversity rather than as a test of HWE for the reasons described above.

Multilocus recombinational linkage disequilibrium among SNP markers was tested by calculating the index of association I_A , which compares observed multilocus data against the null hypothesis that alleles from different loci assort independently (for detailed explanations of this analysis see: Agapow & Burt 2001; Xu 2006). The statistic I_A ranges from zero (no linkage) to a positive statistically non-zero number (linkage disequilibrium). Because this value can vary with the number of loci analyzed, comparisons across studies can be problematic, and significance values are computed by comparisons with a randomized dataset. This analysis was computed using the program MULTILOCUS (Agapow & Burt 2001).

The proportion of polymorphic loci was calculated as the percentage of loci with two alleles within each *shiro*. This statistic was calculated by pooling all *shiro* from each forest age class.

Expected mean genotype diversity, the likelihood that two randomly selected individuals have different multilocus genotypes, was calculated in MULTILOCUS using 999 randomly combined locus permutations (Fig 3A).

To determine whether multilocus genotypes arose from sexual recombination or from somatic mutation associated with vegetative reproduction, a pair-wise codominant genetic distance matrix was calculated using GenAleX 6 (Peakall & Smouse 2006) as in Peakall et al. (1995). For a single-locus analysis, with *i*-th, *j*-th, *k*-th and *l*-th different alleles, a set of squared distances is defined as $d_2(ii, ii) = 0$, $d_2(ij, ij) = 0$, $d_2(ii, ij) = 1$, $d_2(ij, ik) = 1$, $d_2(ij, kl) = 2$, $d_2(ii, jk) = 3$, and $d_2(ii, jj) = 4$. The frequency distributions of these distances were plotted in histograms (as in Arnaud-Haond et al. 2007; Douhovnikoff & Dodd 2003; Rogstad et al. 2002, Fig 3B). Were populations to exhibit only sexual recombination we would expect this distribution to be unimodal and normally distributed. Conversely, where novel genotypes result from somatic mutations in vegetative reproduction, we would expect this distribution to be bi-modal: one peak close to zero

Table 2 – *T. matsutake* mycorrhiza multilocus genotypes

	n	Clone-corrected n	Shiro	Genotypes	Observed genotype diversity	Observed genotype diversity clone-corrected	Mean observed genotype diversity
Jidi OG	9	9	5	8	0.89	0.89	0.78/0.88
Weng OG	18	15	5	13	0.72	0.87	
Jidi 50	6	6	3	6	1.00	1.00	0.66/0.78
Weng 50	26	21	7	15	0.58	0.71	

Sample sizes are shown before and after identical multilocus genotypes originating from a single *shiro* were removed (clone-corrected). Mean observed genotype diversity was calculated by pooling samples from each age class, both including clones and clone-corrected (separated by a slash).

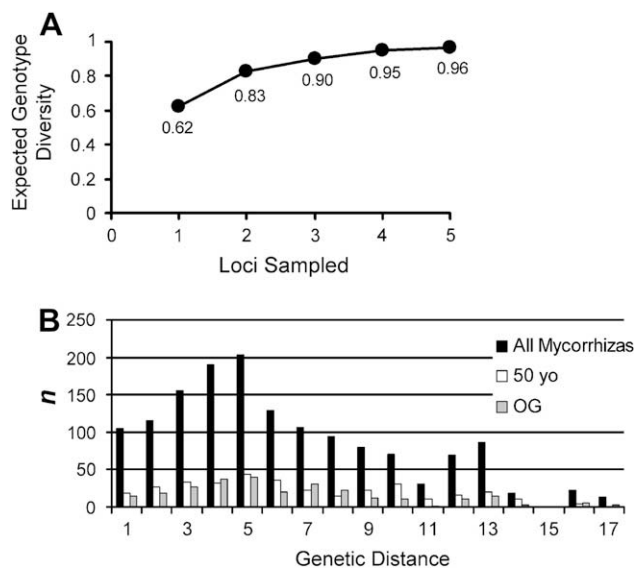


Fig 3 – Mean expected genotype diversity and frequency distribution of pair-wise genetic distance between mycorrhiza. The probability that two randomly sampled individuals will have a different multilocus genotype increases with the number of sampled loci as calculated via a random permutation analysis with 100 repetitions (A). Maximum genotype diversity was 0.96 when all five loci were sampled. Standard deviation for each value was calculated, but was too small (0–0.06) to plot on the graph. Data is from all mycorrhizas sampled in the study. Frequency distributions of pair-wise genetic distance (B) between mycorrhizas within forest ages and combining all data sets were unimodal and normally distributed indicating that somatic mutation did not likely contribute to designation of unique multilocus genotypes in this study.

representative of clonal individuals with slight genetic dissimilarity, and a second peak representative of different recombinant genets.

Analysis of molecular variance (AMOVA) using codominant allelic distances was conducted in GenAlEx 6, using the method of Weir and Cockerham (1984) with 999 permutations. AMOVA analyses are useful for delineating the partitioning of molecular variance to determine at what point, within a hierarchical framework, differentiated genetic structure is detectable. The absence of detectable genetic structure may be expected in continuous populations where there is no significant barrier to gene flow. Here, genetic structure of *T. matsutake* within and between Jidi and Wengshue forests were tested for each age class separately and combined. In this case, these tests were used to determine deviations from the population as a whole, and not population structure *per se*, since the conditions of random sampling and HWE were not met.

The spatial autocorrelation series of tests provides correlations of genetic similarity between individuals located within defined distance classes. We selected distance classes that produced even sample sizes of pair-wise comparisons across classes. For that reason, distance classes within and between the two forest ages are not equal (Fig 4). For each distance class, a correlation coefficient is calculated ranging from –1 to 1. Confidence intervals for significant autocorrelation are calculated with 999 permutations, and additional significance levels are calculated with 10 000 bootstrap resamples. Because genotyped individuals within forest age classes were spatially heterogeneous, we did not use the option for integrating multiple populations in GenAlEx 6, since certain distance classes would then have a sample size of zero. Instead pair-wise distance comparisons within forest age classes were pooled into a single analysis.

Previous studies have demonstrated that the results of single correlograms may be equivocal since the analysis can vary with distance size classes chosen and sample size (Double

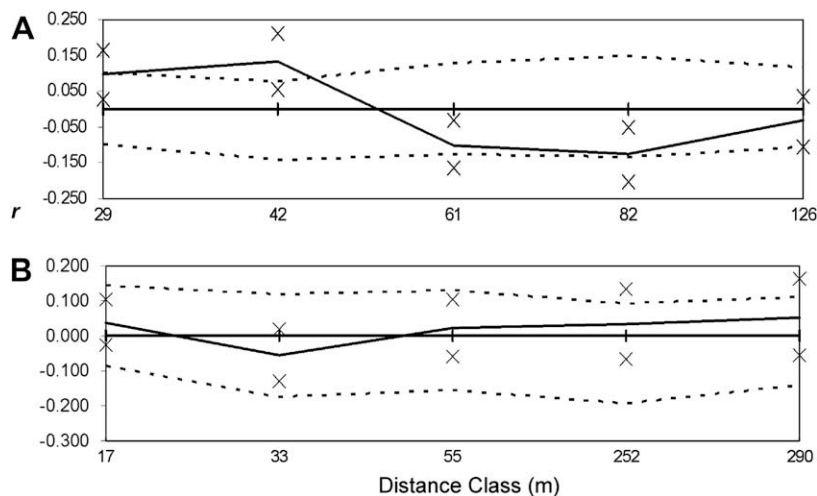


Fig 4 – Correlograms plotting mean spatial autocorrelation for *T. matsutake* mycorrhizas collected in 50-y-old (A), and old-growth forests (B). R values are black circles, dashed lines are 95 % upper and lower confidence levels (999 permutations), Xs are 95 % bootstrap errors (10 000 bootstrap resamples). Bonferonni-corrected *p* values are significant in 50-y-old forests up to the 42 m distance class with an X intercept at 54 m. There is no evidence for spatial autocorrelation in old-growth forests. Notice that X axis scales differ. Identical genotypes from the same putative shiro were excluded from this analysis.

et al. 2005; Peakall et al. 2003). This uncertainty may be particularly acute in this study where sample sizes within each distance were small. An alternative approach is to test increasing distance class sizes for genetic correlation. Here the distance class size where genetic correlation is no longer detected may be interpreted as the full spatial extent of autocorrelation (Dunham et al. 2006; Peakall et al. 2003). This additional test was conducted using the Multiple Distance Class option in GenAlix 6. This analysis is similar to a Mantel's tests for isolation-by-distance, but provides a more spatially-nuanced model of how increased geographic distance classes and sample sizes affects the correlation between geographic distance and genetic dissimilarity (Peakall et al. 2003). Here we increased our distance classes in five-m increments, omitting those distance classes where no additional mycorrhizal pairs were added to the analysis, and binning larger distance classes after 100 m (Fig 5). Data pooling and parameter settings were identical to the other spatial autocorrelation tests described above.

Results

Mycorrhiza genotypes

DNA from 356 mycorrhizas was extracted, and 121 amplified for at least one locus. Only isolates in which all loci amplified were included in analysis: 27 mycorrhizas from 10 shiro (15 soil cores) from 50-y-old forests, and 32 mycorrhizas from 10 shiro (15 soil cores) from old-growth forests (Table 2, Fig 2). Hardy Weinberg equilibrium was found within each forest age class, but not among all of the mycorrhizas when pooled. Both the percentage of polymorphic loci as well as genotype diversity were higher in old-growth compared to 50-y-old forests (Table 3). The I_A did not significantly differ from the null hypothesis that alleles were randomly associated. Mean genotype diversity, the probability that the genetic markers distinguish multilocus genotypes from two randomly sampled

individuals was calculated at 0.96 for all mycorrhizas combined (Fig 3A). Frequency distributions of genetic distance within and between treatments were unimodal and normally distributed (Fig 3B). Thirty-three multilocus genotypes were identified from all isolates.

After clone correction within *shiro*, 10 genotypes were found in more than one *shiro*, eight of those were found in both Wengxue and Jidi forests. Five genotypes were duplicates, three genotypes were found in three *shiro*, one genotype was found in four *shiro* and the most cosmopolitan genotype was represented in five *shiro*: two in Jidi's old-growth forest, and three in Wengshue's 50-y-old forest. Because our molecular markers were clearly insufficient to distinguish genets, we did not attempt to map individuals in space or to infer clone size.

Population spatial genetic structure

Mycorrhizas were positively autocorrelated within the first two distance classes (0–29 and 30–42 m) of 50-y-old forests, with all subsequent distance classes statistically non-different from zero. None of the distance classes of old-growth forests showed that mycorrhizas were significantly autocorrelated (Fig 4).

The multiple distance class correlation analysis demonstrated that 50-y-old forests show a clear pattern of isolation-by-distance, with positive correlation values gradually declining as distance classes increase (Fig 5). Old-growth forests show no detectable correlation at any distance class.

AMOVA

Analysis of molecular variance (AMOVA) demonstrated differences in variance partitioning between forest age classes (Table 4). Both forest age classes show the majority of variance within sites: 100 % in old-growth, and 89 % in 50-y-old, with the remainder between sites. There was no significant

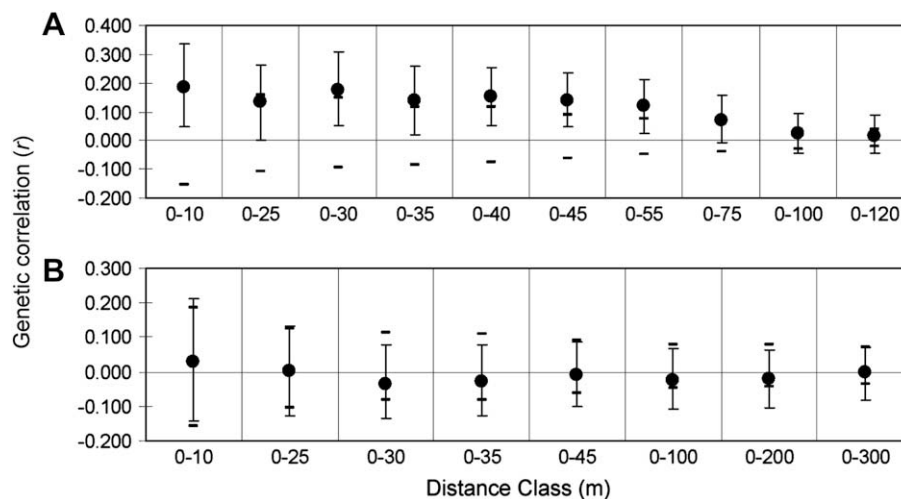


Fig 5 – Genetic correlation within 50-y-old forests (A) and old-growth forests (B) for increasing distance classes. Whiskers are 95 % confidence intervals about random distribution of *T. matsutake* genotypes, error bars are 95 % confidence intervals calculated via bootstrapping. Fifty year-old forests show declining correlations indicative of isolation-by-distance spatial patterning. In old-growth forests, no distance classes are significantly correlated.

Table 3 – Population genetic statistics of *T. matsutake* mycorrhizas pooled by forest age class

	H_o	H_e	I_A	p	Polymorphic loci
OG	0.350	0.383	0.430	0.724	0.88
Std.	0.046	0.026	0.060		0.08
50-y-old	0.236	0.299	0.360	0.331	0.78
Std.	0.036	0.029	0.060		0.07

All statistics are calculated by pooling forest age classes, except for polymorphic loci: the percentage of loci within each shiro with two alleles. H_o is observed heterozygosity, H_e is expected heterozygosity. p value is reported for I_A ; neither age class showed a significant I_A .

variance between villages with samples from both forest age classes pooled.

Discussion

In this study, we have demonstrated that *T. matsutake* population structure is congruent with a sexual reproduction strategy. In both treatments, insignificant I_A and high genotypic diversity indices are indicative of outcrossing populations. This is consistent with other recent studies showing high levels of heterozygosity and recombination in *T. matsutake* at small spatial scales (Murata et al. 2005; Xu et al. 2008). AMOVA analyses indicate that the majority of molecular variance is partitioned within forest age classes, also strongly indicative of outcrossing.

We also demonstrated that the genetic structure of fungal populations may be correlated with forest age. Fifty year-old forests showed lower observed heterozygosity, genotypic diversity and proportion of polymorphic loci per shiro compared to old-growth forests. These differences may be partially explained by spatial autocorrelation patterns. Fifty year-old forest correlograms demonstrated positive significant autocorrelation up to 42 m, followed by values not significantly different from zero. Old-growth forests, conversely, do not demonstrate spatial autocorrelation at any distance.

These results are difficult to compare with spatial autocorrelation findings from other EMF studies, since different

Table 4 – Analysis of molecular variance (AMOVA) results in 50-y-old forests, old-growth forests and pooled samples from both forest ages

Source	d.f.	Sum squares	Mean squares	% variance	F_{st}	p
50-y-old						
Between villages	1	3.502	3.502	11	0.106	0.020
Within villages	62	65.654	1.059	89		
Old-growth						
Between villages	1	0.426	0.426	0	-0.027	0.815
Within villages	52	60.000	1.154	100		
Combined samples						
Between villages	1	1.925	1.925	2	0.010	0.170
Within villages	116	129.456	1.116	98		

Fifty year-old forests show significant partitioning of molecular variance between sites (11%), whereas no significant variance was found between sites in old-growth forests or between sites where individuals from both forest ages were pooled.

spatial scales and analyses were used. Kretzer et al. (2005) did not detect spatial autocorrelation in either of two sister *Rhizopogon* spp. sampled in 40–80-y-old conifer forests with genetic clones removed from their data set. Hypogeous *Rhizopogon*, as the authors note, is largely mammal-dispersed and not, therefore, subject to the same dispersal constraints as wind-dispersed epigeous fungi. Conversely, Dunham et al. (2006) found that genets of *Cantherellus formosus* were significantly autocorrelated up to and beyond 120 m in a 50-y-old conifer forest. Their multiple distance class correlation analysis shows the full spatial extent of spatial genetic structure at approximately 400 m. Most recently, Carriconde et al. (2008) found maximum spatial autocorrelation correlogram X axis intercept values at distances up to 20.3 and 6.3 m among two genetic groups of *Tricholoma scalpuratum*. Two replications among sites in this study showed a fair amount of variation. Their findings of small-scale population structure and gene flow restriction seems most consistent with our results from 50-y-old forests.

Our frequency distribution analysis of pair-wise genetic distance indicates that diversity detected within populations is not likely generated by somatic mutations. Gryta et al. (2000) used a similar analysis to correct for what was determined to be overestimation of *Hebeloma cylindrosporum* genets in a previous study. The sensitivity of our analysis, however, is directly proportional to the discriminatory power of our molecular markers, and it is unlikely that we would detect low-level somatic mutations in this study. Although the permutation analysis of expected mean genotype diversity predicted a 0.96 probability of distinguishing randomly selected individuals from a population, this analysis assumes that individuals are not related, an assumption clearly violated in 50-y-old forests where genotypic spatial autocorrelation was found <42 m.

One hypothesis to explain the differences found between forest age classes is that *T. matsutake* structure in younger forests is the result of a localized founder effect. If infection rate is correlated with spore load, we would suspect that a high proportion of novel infections are caused initially by local sources of inoculum. This ought to result in positive correlations between genetic and geographic distance reflecting steeply declining spore abundance as proximity to parent sporocarps decreases. This pattern is evident in 50-y-old forests in which significant spatial autocorrelation and isolation-by-distance patterns are found. Mating-type genes conferring sexual incompatibility are known to reduce inbreeding in some EMF populations, although even in tetrapolar mating-type systems “sibling” compatibility remains at 25% (Moore & Novak Fraser 2002). Likewise, in nature, secondary homothallism may still occur in predominantly outcrossing species (Bonello et al. 1998). To our knowledge, the mating-type system in *T. matsutake* is unknown.

Over time, an accumulation of spores originating from far enough away so as not to autocorrelate with indigenous genotypes (42 m in this study) may germinate inside shiro and dilute spatial patterning. Because *T. matsutake* shiro are comprised of multiple genotypes, the accumulation of new, unrelated individuals increases with time.

Other evidence for a shift from localized founder effect to a long-distance-mediated reproductive dynamic is provided

by higher levels of homozygosity within 50-y-old forests compared to old-growth. Furthermore, AMOVA analysis indicates that 50-y-old forests have significant F_{st} values (0.106), indicating that forests in the two villages (separated by 60 km) show somewhat restricted gene flow compared to old-growth forests separated by the same distance. Genetic dissimilarity between the 50-y-old forests could be the result of a founder effect and subsequent colonization by localized spore rain. It should be noted that spatial autocorrelation should be accounted for when collecting fungi from different habitats to test for landscape-level population genetic variation, since overestimating population substructure could be an artifact of collecting related individuals (Diniz-Filho & De Campos Telles 2002; Dunham et al. 2006).

Because closely related individuals are more difficult to distinguish than those more distantly related, our clone-corrected analyses likely under-represented the genotype diversity of closely related individuals. Identical multilocus genotypes, identified here as clones, were removed from within *shiro* for most analyses, so this bias was spatially dependent in our study. This may have introduced biases into our spatial autocorrelation analyses and contributed to the lack of spatial structure detected in old-growth forests. Conversely, "clones" from within *shiro* were included in AMOVA analyses. Therefore the possibility exists that significant molecular variance found between Wengshue and Jidi 50-y-old forests could be an artifact of repeatedly sampling the same individual.

Conversely, in old-growth forests, gene-flow is less restricted among villages, as evidenced by lack of structure in AMOVA analyses. Recruitment in these populations is evidently from more distant sources, resulting in allele frequencies non-different from the population as a whole. We hypothesize that this shift from local to more distant sources of inoculum is not reflective merely of spore load *per se*, since local spores will almost certainly outnumber those dispersed over long distances in any population by orders of magnitude. Instead, we suggest that founder effects are diminished by gradually accumulating novel genotypes over time. Furthermore, this process may be facilitated by aspects of *T. matsutake* autoecology.

There is some evidence to suggest that *T. matsutake* establishment may be density dependent. Murata et al. (2005) were only able to germinate *T. matsutake* spores when they were densely packed around mushroom gills or treated with *n*-butyric acid. This, and the fact that no *T. matsutake* mycorrhizas are found outside of *shiro* (Amend unpublished data; Lian et al. 2006) may indicate that conditions created by the dense hydrophobic mycelia mat within *shiro* are favorable to *T. matsutake* basidiospore germination. Cultures of the closely related *T. magnivelare* have been found to produce two chlorinated compounds: 3,5-dichloro-4-methoxybenzaldehyde and 3,5-dichloro-4-methoxybenzyl alcohol, which are known to inhibit chitin and melanin synthesis, molecules which play important roles in fungal cell wall structure and defense (Wood & Lefevre 2007). This may be one mechanism by which fairy-ring-forming *Tricholoma* competitively exclude other species.

In this case, while few pioneering genotypes successfully establish in the absence of previously established genotypes of *T. matsutake*, increasing *shiro* size and colonized area

creates more available space for facilitated basidiospore establishment. In older forests this could facilitate establishment of spore rain originating from distant populations and could explain the difference in spatial dynamics encountered between the two forest ages studied. Mating-type systems which favor the establishment of exogenous spores would be consistent with this pattern.

Genetic mosaicism via spore dispersal could be the result of either di-mon mating between dikaryotic mycelia associated with the *shiro* and novel monokaryotic mycelia from germinated spores, or convergent dikaryotic fairy rings. Ogawa (1975b) observed multiple small *shiro* expanding until they converged and formed a single, larger, *shiro*. This observation was supported by Lian et al. (2006) study which found that multiple within-*shiro* genets were tightly aggregated and non-overlapping. Alternatively di-mon mating has been found in natural populations of the saprotrophic basidiomycetes *Heterobasidion* spp., *Armillaria gallica* and *A. ostayae* (Garbelotto et al. 2004; Peabody et al. 2000; Rizzo & May 1994). Notably, Murata et al.'s (2005) study of *T. matsutake*, found heterologous genotypes when comparing mushrooms to their single-spore isolates. This provides some evidence that vegetative incompatibility systems may not prohibit anastomoses of non-self mycelium, although the full extent of this phenomenon has yet to be tested. Further evidence could be found by finer-scale examination of intra-specific variation, or by examining the genotypic diversity of extramatrical mycelium in *shiro*. Polymorphic markers with multiple alleles known to assort under Mendelian patterns of inheritance could help discern the prevalence of di-mon mating in natural populations of *T. matsutake*. Alternatively, haplotypes of individuals could be cloned and analyzed for evidence of multiple progenitors.

Our own efforts to this end were hampered by a low number of mycorrhizas successfully genotyped. Our difficulty amplifying DNA from mycorrhizas highlights one of the more obvious pitfalls of working with small amounts of tissue to target DNA loci of single or low-copy number. The low sample size ultimately realized prohibited more detailed analyses of gene-flow, and increased the likelihood of erroneous conclusions regarding the extent of spatial genetic patterns.

In conclusion, it appears that although airborne meiospores are generally responsible for the establishment of new genotypes of this important mycorrhizal fungus, a significant difference in success of fungal establishment may exist between young and old forests. We suggest that this difference, characterized by increasing success as forests age, may depend on whether *T. matsutake* may already be established in a site or not. In the absence of previously established genotypes, local spore-producing fruit bodies may be responsible for initial establishment of this fungus. Secondly, germination facilitated by established genotypes allows for establishment of spores produced by non-local individuals. This complex population dynamic strategy may allow for the maintenance of local well adapted genotypes and of a sufficiently large gene pool, a necessary resource to avoid excessive bottleneck and genetic drift phenomena. This study raises several questions regarding how fungal mating systems, chemical ecology, and other cryptic autoecological factors govern intra-population dynamics.

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