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Evidence for strong inter- and intracontinental phylogeographic structure in *Amanita muscaria*, a wind-dispersed ectomycorrhizal basidiomycete

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ABSTRACT

A growing number of molecular studies show that many fungi have phylogeographic structures and that their distinct lineages are usually limited to different continents. As a conservative test of the extent to which wind-dispersed mycorrhizal fungi may exhibit phylogeographic structure, we chose to study *Amanita muscaria*, a host-generalist, widespread, wind-dispersed fungus. In this paper, we document the existence of several distinct phylogenetic species within *A. muscaria*, based on multilocus DNA sequence data. According to our findings, *A. muscaria* has strong intercontinental genetic disjunctions, and, more surprisingly, has strong intracontinental phylogeographic structure, particularly within North America, often corresponding to certain habitats and/or biogeographic provinces. Our results indicate that the view of *A. muscaria* as a common, widespread, easily identifiable, ecologically plastic fungus with a wide niche does not correctly represent the ecological and biological realities. On the contrary, the strong associations between phylogenetic species and different habitats support the developing picture of ecoregional endemisms and relatively narrow to very narrow niches for some lineages.

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1. Introduction

Mycorrhizal associations are abundant and widespread in almost all ecosystems and approximately 80% of land plant species form associations with mycorrhizal soil fungi (Trappe, 1987). In such symbioses, fungi support plants with mineral nutrients, water and other services and the fungi, in turn, receive photosynthates from the autotrophic plants. Given their abundance and their effects on plant growth, they are known to play important roles in ecosystems (e.g., Read and Perez-Moreno, 2003; Johnson and Gehring, 2007).

The sensitivity of mycorrhizal fungi to climate change is essentially unknown. The ability of an individual fungal species to cope with the changing environment is likely to be related to their genetic diversity. According to the basic principles of conservation genetics, populations possessing a small amount of genetic diversity are more susceptible to regional extinction during times of stress (e.g., rapid climatic change) than genetically diverse populations (e.g., Avise, 2000). Unveiling phylogeographic structures of ectomycorrhizal species, assessing their genetic diversity, and reconstructing their past responses to past climatic changes will help to fill this important void.

As a conservative test of the extent to which wind-dispersed mycorrhizal fungi may exhibit phylogeographic structure, we chose to study *Amanita muscaria* (L.: Fr.) Hooker. *Amanita muscaria* is native to temperate and boreal forest regions of the Northern Hemisphere, where it is an ectomycorrhizal (ECM) fungus with a wide host range (Trappe, 1987). Although it is most commonly associated with various birch (*Betula*), pine (*Pinus*), spruce (*Picea*), fir (*Abies*), and larch (*Larix*) species, it is known to form ECM associations with representatives of other genera, particularly when its primary hosts are rare or non-existent in a certain area. *Amanita muscaria* has traditionally been reported as a single morphospecies, although morphological variation has led to the publication of several intraspecific varieties, such as *A. muscaria var. muscaria* (L.: Fr.) Hooker, *A. muscaria var. alba* Peck, *A. muscaria var. flavivolvata* (Singer) Jenkins, *A. muscaria var. formosa* (Pers.: Fr.) Bertillon in DeChambre, *A. muscaria var. persicina* Jenkins, and *A. muscaria var. regalis* (Fr.) Bertillon in DeChambre (Jenkins, 1986).

This well known fungus is predicted to have little biogeographic structure for the following reasons: (1) it is widely distributed and abundant, (2) its spores are largely wind-dispersed and it produces copious above-ground fruiting bodies (mushrooms), (3) it associates with a wide variety of both coniferous and angiosperm host trees, and thus appears to have little host-specificity, and (4) it is considered to be an invasive species where it has been introduced in the Southern Hemisphere (Bagley and Orlovich, 2004).

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Our findings, reported herein, are in sharp contrast to these theoretical predictions. Here, we document the existence of several distinct phylogenetic species within *A. muscaria*, based upon multilocus sequence data and various phylogenetic and population genetic analyses. We find that *A. muscaria* has strong intercontinental genetic disjunctions, and, more surprisingly, shows strong phylogeographic patterns within North America. Our results indicate that the view of *A. muscaria* as a common, widespread, easily identifiable, ecologically plastic fungus with a wide niche does not correctly represent the ecological and biological realities. On the contrary, the strong associations between phylogenetic clades (both at species and intraspecific levels) and different habitats support the developing picture of ecoregional endemisms and relatively narrow to very narrow niches for some lineages.

2. Materials and methods

2.1. Isolates and DNA extraction

Ninety-eight specimens were collected from various geographic regions spanning the known distribution of *A. muscaria* (Table 1). DNA was extracted from small samples of dried specimens using the E-Z 96 Fungal DNA Kit (Omega Bio-tek, Inc., Doraville, GA) or the DNeasy Plant Mini Kit (QIAGEN, Inc., Valencia, CA).

2.2. PCR and DNA sequencing

DNA sequence data were obtained for four loci: *β-tubulin* gene, translation elongation factor 1-alpha gene (*EF1-α*), nuclear large ribosomal subunit gene (LSU), and the internal transcribed spacer (ITS) + 5.8S ribosomal subunit gene region. The primers, PCR, and sequencing protocols have been described previously (Geml et al., 2005, 2006). The only exception was the *β-tubulin* gene, for which a new primer pair was constructed to specifically amplify and sequence an approximately 180-bp fragment containing the most informative known region within *β-tubulin* in *A. muscaria*: AMBT-F (5′ CAA AGC GGA GCA GGT AAT AA) and AMBT-R (5′ AGT ACC GCC ACC AAG CGA AT).

2.3. Phylogenetic analysis

Sequence data obtained for both strands of each locus were edited and assembled for each isolate using CodonCode Aligner v. 1.3.4 (CodonCode Inc., Dedham, MA). Newly generated sequences were deposited in Genbank (EU071826–EU072015). Additional, previously published (Oda et al., 2004; Geml et al., 2006) *A. muscaria* DNA sequences were included in the analyses (Table 1). Homologous sequences of *Amanita pantherina* (isolate FB-30958) (Oda et al., 2004) were used to root all trees. Sequence alignments were initiated using Clustal W (Thompson et al., 1997) and subsequently corrected manually. To test for phylogenetic conflict among the different loci (i.e., if individual gene trees significantly differed from each other), the partition homogeneity test (PHT) was performed with 1000 randomized datasets, using heuristic searches with simple addition of sequences in PAUP* 4b10 (Swofford, 2002). Analyses were conducted using maximum-parsimony (MP) and maximum-likelihood (ML) methods in PAUP* and Garli 0.94 (Zwickl, 2006), respectively. For the latter, the best-fit evolutionary model was determined by comparing different evolutionary models with varying values of base frequencies, substitution types, α-parameter of the γ-distribution of variable sites, and proportion of invariable sites via the Akaike information criterion (AIC) using PAUP* and Modeltest 3.06 (Posada and Crandall, 1998). Gaps were scored as 'missing data'. Bootstrap (B) test (Felsenstein, 1985) was used with 1000 replicates in both MP and ML, with the maximum number of trees saved set to 10 for each replicate. To compare different tree topologies, Shimodaira–Hasegawa tests were used (Shimodaira and Hasegawa, 1999). The High Performance Computing cluster maintained by the UAF Biotechnology Computing Research Group (http://biotech.inbre.alaska.edu/) was used to run Clustal W, Modeltest, and Garli.

2.4. Polymorphism and divergence

The number of polymorphic sites and their distribution among the major clades was determined for sequence data generated from all loci (ITS, *β-tubulin*, *EF1-α*, LSU). Within species, nucleotide diversity was measured using π, the average number of nucleotide differences among sequences in a sample (Nei and Li, 1979). Between species, divergence was measured as D_{xy}, the average number of nucleotide substitutions per site between species pairs (Nei and Kumar, 2000). In addition, genetic differentiation (*Fst*) (Hudson et al., 1992), the number of fixed differences, and shared mutations were calculated for the species pairs, as were the number of positions that were polymorphic in one phylogenetic species but monomorphic in the other. Measures of variation and differentiation were performed with the computer program DnaSP v. 4.10.9 (Rozas and Rozas, 1999).

2.5. Genetic differentiation among populations within phylogenetic species

Because the phylogenetic species mentioned are non-interbreeding entities, the population-level analyses were conducted separately for each species clade that contained enough specimens with intraspecific variation: namely, clades I and II. Identical sequences were collapsed into haplotypes using SNAP Map (Aylor et al., 2006) after excluding insertion or deletions (indels) and infinite sites violations. The analyses presented here assume an infinite sites model, under which a polymorphic site is caused by exactly one mutation and there can be no more than two segregating bases. Site compatibility matrices were generated from each haplotype dataset using SNAP Clade and Matrix (Bowden et al., 2008) to examine compatibility/incompatibility among all variable sites, with any resultant incompatible sites removed from the data set. Tajima's D (Tajima, 1989) and Fu and Li's D^* and F^* (Fu and Li, 1993) test statistics were calculated with DnaSP v. 3.53 (Rozas and Rozas, 1999) to test for departures from neutrality. Genetic differentiation among geographic populations was analyzed using SNAP Map, Seqtomatrix, and Permtest (Hudson et al., 1992) implemented in SNAP Workbench (Price and Carbone, 2005). Permtest is a non-parametric permutation method based on Monte Carlo simulations that estimates Hudson's test statistics (*KST*, *KS*, and *KT*) under the null hypothesis of no genetic differentiation. For this purpose, specimens in clade I were assigned to the 'Alaskan', 'Eastern North American', 'Western North American', or 'Mexican' groups based on the geographic regions they occupied. In clade II, specimens were assigned to the 'Alaskan', 'European', 'Asian', and 'Pacific NW North American' groups, the latter representing clade II/A in Fig. 1. Significance was evaluated by performing 1000 permutations. If we found evidence for geographic subdivision, MDIV (Nielsen and Wakeley, 2001) was used to determine whether there was any evidence of migration between pairs of subdivided populations. MDIV implements both likelihood and Bayesian methods using Markov chain Monte Carlo (MCMC) coalescent simulations to estimate the migration rate (*M*), population mean mutation rate (*Theta*), and divergence time (*T*). Ages were measured in coalescent units of 2*N*, where *N* is the population size. This approach assumes that all populations descended from one panmictic population that may or may not have been followed by migration.

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