Molecular Phylogenetics and Evolution 48 (2008) 694–701

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

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

József Geml [a,*], Rodham E. Tulloss [b,1], Gary A. Laursen [a], Nina A. Sazanova [c], D.L. Taylor [a]

[a] Institute of Arctic Biology, University of Alaska Fairbanks, 311 Irving I Building, 902 N. Koyukuk Drive, P.O. Box 757000, Fairbanks, AK 99775-7000, USA
[b] P.O. Box 57, Roosevelt, NJ 08555-0057, USA
[c] Laboratory of Botany, Institute of Biological Problems of the North, Far East Branch of Russian Academy of Sciences, Portovaya Street 18, Magadan 685000, Russia

ARTICLE INFO

Article history:
Received 20 November 2007
Revised 18 April 2008
Accepted 22 April 2008
Available online 29 April 2008

Keywords:
Amanita muscaria
β-tubulin gene
Fungi
Internal transcribed spacer region
Phylogeography
Ribosomal large subunit gene
Translation elongation factor 1-alpha gene

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.

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).

* Corresponding author. Fax: +1 907 474 6967.
E-mail address: jgeml@iab.alaska.edu (J. Geml).
[1] Res. Assoc. (hons.), New York Botanical Garden, Bronx, NY, USA.

1055-7903/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2008.04.029

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.

Table 1
Amanita muscaria isolates included in the multilocus phylogenetic analyses

Isolate code	Origin	GenBank Accession Number			
		ITS	*β-tubulin*	LSU	*EF1-α*
GAL2005	Bonanza Creek LTER site, Alaska, USA	EU071947	–	EU072006	–
GAL2810	Dalton Hwy, mile 122, Alaska, USA	DQ060904	EU071849	DQ060884	EU071872
GAL2814	Dalton Hwy, mile 122, Alaska, USA	DQ060897	DQ060917	DQ060877	–
GAL3169	Eagle Summit, Alaska, USA	DQ060905	EU071850	DQ060885	–
GAL3643	Glacier Hwy, mile 30, Juneau, Alaska, USA	EU071948	–	EU072007	–
GAL3688	Glacier Hwy, mile 27, Juneau, Alaska, USA	DQ060906	–	DQ060886	–
GAL4247	Glacier Hwy, mile 27, Juneau, Alaska, USA	DQ060894	DQ060914	DQ060874	EU071870
GAL4302	Glacier Hwy, mile 27, Juneau, Alaska, USA	DQ060910	DQ060923	DQ060890	EU071871
GAL4810	Denali National Park, Alaska, USA	EU071937		EU071996	–
GAL5505	Denali National Park, Alaska, USA	DQ060908	DQ060922	DQ060888	–
GAL5895	Serpentine Hot Springs, N. of Nome, Alaska, USA	DQ060898	DQ060918	DQ060878	–
GAL5900	Serpentine Hot Springs, N. of Nome, Alaska, USA	DQ060902	–	DQ060882	–
GAL5946	Quartz Creek, E. of Nome, Alaska, USA	DQ060903	–	DQ060883	–
GAL6027	Kougarok Rd., mile 49, Nome, Alaska, USA	DQ060909	EU071851	DQ060889	EU071873
GAL8950	Denali National Park, Alaska, USA	DQ060901	–	DQ060881	–
GAL14284	Denali National Park, Alaska, USA	DQ060895	DQ060915	DQ060875	–
GAL15330	Fairbanks, Alaska, USA	DQ060891	DQ060911	DQ060871	EU071869
GAL15335	Fairbanks, Alaska, USA	EU071945	–	EU072004	–
GAL15336	Fairbanks, Alaska, USA	EU071906	EU071846	EU071979	–
GAL15453	North Pole, Alaska, USA	DQ060899	DQ060919	DQ060879	–
GAL15454	North Pole, Alaska, USA	EU071943	–	EU072002	–
GAL15461	North Pole, Alaska, USA	DQ060900	DQ060920	DQ060880	–
GAL15776	Bonanza Creek LTER site, Alaska, USA	DQ060893	DQ060913	DQ060873	–
GAL16654	Fairbanks, Alaska, USA	DQ060907	DQ060921	DQ060887	–
GAL16735	Fairbanks, Alaska, USA	DQ060896	DQ060916	DQ060876	–
GAL16775	Fairbanks, Alaska, USA	DQ060892	DQ060912	DQ060872	–
GAL17647	Fairbanks, Alaska, USA	EU071907	EU071847	EU071980	–
GAL17691	Fairbanks, Alaska, USA	EU071956	–	EU072015	–
GAL17899	Fairbanks, Alaska, USA	EU071950	–	EU072009	–
GAL17982	Fairbanks, Alaska, USA	EU071938	–	EU071997	–
GAL17984	Fairbanks, Alaska, USA	EU071908	EU071848	EU071981	–
GAL18071	Fairbanks, Alaska, USA	EU071940	–	EU071999	–
GAL18076	Fairbanks, Alaska, USA	EU071942	–	EU072001	–
GAL18122	Bonanza Creek LTER site, Alaska, USA	EU071944	–	EU072003	–
GAL18134	Bonanza Creek LTER site, Alaska, USA	EU071946	–	EU072005	–
GAL18136	Bonanza Creek LTER site, Alaska, USA	EU071949	–	EU072008	–
GAL16735-2	Fairbanks, Alaska, USA	EU071939	–	EU071998	–
GAL16735-3	Fairbanks, Alaska, USA	EU071941	–	EU072000	–
GAL18012-2	Fairbanks, Alaska, USA	EU071952	–	EU072011	–
GAL18012-4	Fairbanks, Alaska, USA	EU071953	–	EU072012	–
GAL18012-6	Fairbanks, Alaska, USA	EU071951	–	EU072010	–
GAL18810-1	Homer, Alaska, USA	EU071955	–	EU072014	–
GAL18810-2	Homer, Alaska, USA	EU071954	–	EU072013	–
RET024-3	Harrison Co., Mississippi, USA	EU071886	EU071826	EU071963	–
RET032-1	Essex Co., Massachusetts, USA	EU071890	EU071830	EU071967	–
RET036-3	Zürich canton, Switzerland	EU071912	–	EU071985	–
RET107-6	Highlands & Islands Reg., Scotland, UK	EU071909	–	EU071982	EU071874
RET112-5	Harrison Co., Mississippi, USA	EU071887	EU071827	EU071964	EU071859
RET112-6	Harrison Co., Mississippi, USA	EU071888	EU071828	EU071965	–
RET124-2	Suffolk Co., Massachusetts, USA	EU071896	EU071836	–	–
RET136-2	Whatcom Co., Washington, USA	EU071936	–	–	EU071878
RET141-2	Highlands & Islands Reg., Scotland, UK	EU071918	–	EU071991	–
RET143-5	Kamchatka, Russia	EU071915	–	EU071988	–
RET144-10	Tlaxcala, Mexico	EU071913	–	EU071986	–
RET145-1	Tlaxcala, Mexico	EU071921	–	EU071994	EU071876
RET145-2	Tlaxcala, Mexico	EU071903	EU071843	EU071978	EU071866
RET149-1	Southern Highlands Prov., Tanzania	EU071895	EU071835	EU071971	–
RET149-2	Southern Highlands Prov., Tanzania	EU071894	EU071834	–	–
RET151-4	Talladega Co., Alabama, USA	EU071892	EU071832	EU071969	EU071862
RET151-6	Shelby Co., Alabama, USA	EU071891	EU071831	EU071968	EU071861
RET152-6	Baden-Württemberg, Germany	EU071897	EU071837	EU071972	–
RET152-8	Bavaria, Germany	EU071920	–	EU071993	–
RET158-7	Burlington Co., New Jersey, USA	EU071916	–	EU071989	–
RET264-7	Skamania Co., Washington, USA	EU071898	EU071838	EU071973	–
RET271-2	Sussex Co., New Jersey, USA	EU071899	EU071839	EU071974	EU071864
RET271-3	Somerset Co., New Jersey, USA	EU071919	–	EU071992	–
RET289-3	Cape May Co., New Jersey, USA	EU071901	EU071841	EU071976	–
RET303-4	Monmouth Co., New Jersey, USA	EU071917	–	EU071990	EU071875
RET309-3	Sogn og Fjordane, Norway	EU071914	–	EU071987	–
RET320-1	Fremont Co., Idaho, USA	EU071911	–	EU071984	–
RET328-2	Sussex Co., New Jersey, USA	EU071926	–	EU071995	EU071877
RET338-9	Clallam Co., Washington, USA	EU071900	EU071840	EU071975	–
RET383-3	Newfoundland, Canada	EU071893	EU071833	EU071970	EU071863
CMP 0648	Cochise Co., Arizona, USA	EU071910	–	EU071983	–

Table 1 (*continued*)

Isolate code	Origin	GenBank Accession Number			
		ITS	*β-tubulin*	LSU	*EF1-α*
CMP 1345	Cochise Co., Arizona, USA	EU071902	EU071842	EU071977	EU071865
CMP 3143	Cochise Co., Arizona, USA	EU071889	EU071829	EU071966	EU071860
NS1	Ola district, Magadan region, Russia	EU071934	–	–	–
NS3	Ola district, Magadan region, Russia	EU071922	–	–	–
NS4	Ola district, Magadan region, Russia	EU071904	EU071844	–	EU071867
NS5	Magadan district, Magadan region, Russia	EU071924	–	–	–
NS6	Magadan district, Magadan region, Russia	EU071927	–	–	–
NS8	Magadan district, Magadan region, Russia	EU071932	–	–	–
NS10	Magadan district, Magadan region, Russia	EU071905	EU071845	–	EU071868
NS11	Ola district, Magadan region, Russia	EU071935	–	–	–
NS12	Ola district, Magadan region, Russia	EU071923	–	–	–
NS13	Omsukchan district, Magadan region, Russia	EU071925	–	–	–
NS14	Tenka district, Magadan region, Russia	EU071928	–	–	–
NS15	Tenka district, Magadan region, Russia	EU071930	–	–	–
NS16	Anadyr district, Chukot Autonomous Region, Russia	EU071933	–	–	–
MP23	Bird Creek Campground, Anchorage, Alaska, USA	EU071929	–	–	–
MP24	Bird Creek Campground, Anchorage, Alaska, USA	EU071931	–	–	–
LG382	Santa Cruz Island, California, USA	EU071957	EU071852	–	EU071879
LG458	Santa Cruz Island, California, USA	–	EU071853	–	EU071880
LG862	Santa Cruz Island, California, USA	EU071958	EU071854	–	EU071881
LG864	Santa Cruz Island, California, USA	EU071959	EU071855	–	EU071882
LG882	Santa Cruz Island, California, USA	EU071960	EU071856	–	EU071883
LG1045	Santa Cruz Island, California, USA	EU071961	EU071857	–	EU071884
LG1066	Santa Cruz Island, California, USA	EU071962	EU071858	–	EU071885
30961*	Aomori-shi, Aomori, Japan	AB080980	AB095892	–	–
30962*	Kitakoma-gun, Yamanashi, Japan	AB080981	AB095893	–	–
30963*	Kitakoma-gun, Yamanashi, Japan	AB080982	AB095894	–	–
30976*	Kiso-gun, Nagano, Japan	AB081294	AB095895	–	–
30977*	Ohno-gun, Gifu, Japan	AB081295	AB095896	–	–
30985*	Ohno-gun, Gifu, Japan	AB096048	AB095897	–	–
30978*	Chino-shi, Nagano, Japan	AB081296	AB095858	–	–
30981*	Chino-shi, Nagano, Japan	AB096049	AB095859	–	–
30982*	Chino-shi, Nagano, Japan	AB096050	AB095860	–	–
30964*	Gdynia, Poland	AB080983	AB095899	–	–
30965*	Gdansk, Poland	AB080984	AB095900	–	–
31452*	Hampshire, England, UK	AB080777	AB095901	–	–
31445*	Surrey, England, UK	AB080778	AB095902	–	–
80048*	Surrey, England, UK	AB080779	AB095903	–	–
30987*	Queenstown, New Zealand	AB096052	AB095904	–	–
45843*	Hampshire, Massachusetts, USA	AB080788	AB095884	–	–
45785*	Hampshire, Massachusetts, USA	AB080789	AB095885	–	–
45840*	Lawrence, Massachusetts, USA	AB080791	AB095887	–	–
45820*	Bronx, New York, USA	AB080790	AB095886	–	–
45863*	Mendocino, California, USA	AB080787	AB095883	–	–
49100*	Cascade, Idaho, USA	AB080793	AB095889	–	–
45883*	Piscataquis, Massachusetts, USA	AB080792	AB095888	–	–
45060*	Amador, California, USA	AB080795	AB095891	–	–
44761*	Alpine, California, USA	AB080794	AB095890	–	–
506*	Dovre, Oppland, Norway	AB080780	AB095855	–	–
1539*	Gjøvik, Oppland, Norway	AB080781	AB095856	–	–
4220*	Nordre-land, Oppland, Norway	AB080782	AB095857	–	–
30986*	Aomori-shi, Aomori, Japan	AB096051	AB095898	–	–

Sequences of isolates marked by '*' were published by Oda et al. (2004).

3. Results

3.1. Phylogenetic analysis

The ITS, *β-tubulin*, LSU, *EF1-α*, and the combined datasets consisted of 691, 195, 724, 439, and 2049 characters, respectively, including gaps. There were 35, 21, 18, 35, and 109 parsimony-informative characters, respectively. The PHT indicated that the phylogenetic signals present in the different loci were not in conflict ($P = 0.1146$). The General-Time-Reversible model, with calculated proportion of invariable sites (I = 0.6106) and estimated α-parameter (= 0.9005) of γ-distribution (GTR+I+G), was selected as the best-fit evolutionary model. One of the equally parsimonious trees is shown in Fig. 1. The Shimodaira–Hasegawa test revealed that there were no significant differences among the ML and MP phylograms ($P = 0.164$). The ML phylogram ($-\ln L = 4962.5256$) is published in the Supporting Information (Fig. S1).

Eight major lineages receiving high support (I–VIII, Fig. 1) were detected within *A. muscaria*. All clades were supported by all loci except that clades I, II, and III were not monophyletic in the *β-tubulin* phylogram. Nonetheless, the *β-tubulin* MP tree did not show significant conflict with MP trees generated from the other loci. When the clades were under monophyletic constraint, the most parsimonious *β-tubulin* trees were only 0–2 steps longer than the unconstrained trees described earlier. Apparently, this lack of conflict was not due to low phylogenetic signal in *β-tubulin*. A permutation tail probability (PTP) test (Archie, 1989; Faith and Cranston, 1991) revealed that the *β-tubulin* locus contributes phylogenetic signal to the combined dataset, because tree length of the original *β-tubulin* phylogram was significantly shorter ($P < 0.01$) than the length of the trees generated based on randomly permuted *β-tubulin* datasets. As expected, clades I, II, III, IV, V, and VI were strongly supported in analyses of the combined dataset with 77%, 93%, 81%, 100%,

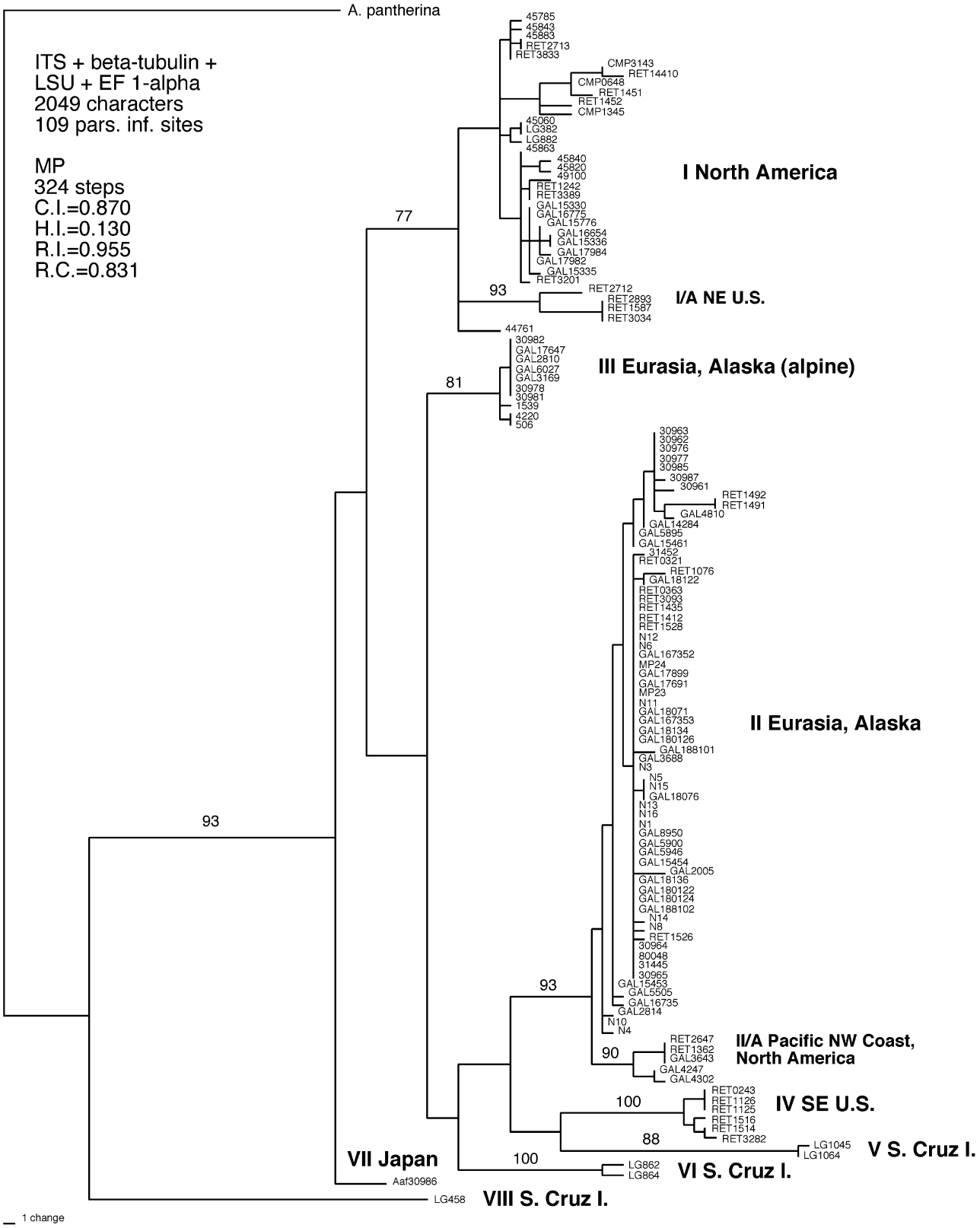

Fig. 1. One of the equally parsimonious trees for the combined dataset with >70% maximum-parsimony bootstrap values shown above the supported branches.

100%, and 88% MPB values, respectively (Fig. 1). Groupings within these major clades were generally not significantly supported with the exception of two subclades: I/A and II/A with 93% and 90% MPB values, respectively. Phylogenetic relationships among the major clades remained unclear, as none of the groupings were significantly supported, except that lineage VIII represents a sister group to the rest of the *A. muscaria* complex, the latter being monophyletic with 93% MPB.

Table 2
Nucleotide polymorphism, known distribution and habitat of the major clades within *A. muscaria*

Species clade	Locus ITS	*β-tubulin*	*EF1-α*	LSU	Known distribution	Ecoregion/habitat	Host
Clade I					Throughout North America,	Temperate and	Various deciduous and
n	34	24	10	22	including the continental US and Alaska	boreal forests	coniferous trees
H	15	10	7	6			
π	0.0035	0.0087	0.0128	0.0027			
Clade II					Europe, Asia, Alaska, Pacific NW	Temperate, boreal,	Various deciduous and
n	70	28	6	41	coast of North America	and coastal forests	coniferous trees
H	12	7	4	3			
π	0.0019	0.0089	0.0060	0.0003			
Clade III					Europe, Asia, Alaska	Subalpine and alpine tundra	*Dryas*, *Salix*
n	10	10	2	4			
H	3	1	1	1			
π	0.0010	0	0	0			
Clade IV					SE North America	Southern mixed forest	*Pinus*, *Quercus*
n	6	5	4	6		(oak–hickory–pine)	
H	1	2	4	1			
π	0	0.0073	0.0033	0			
Clade V					Santa Cruz Island (California)	Chaparral and oak–pine	*Quercus*
n	2	2	2	n.a.		woodlands	
H	2	1	2	n.a.			
π	0.0035	0	0.0020	n.a.			
Clade VI					Santa Cruz Island (California)	Chaparral and	*Lyonothamnus*
n	2	2	2	n.a.		oak–pine woodlands	
H	1	1	1	n.a.			
π	0	0	0	n.a.			

Lineages represented by single specimens (VII and VIII) were excluded from this analysis. Values of the number of sequences (*n*), number of haplotypes (*H*), and the average number of nucleotide differences per site (π) are given.

3.2. Polymorphism and divergence

Intraclade nucleotide polymorphism is summarized in Table 2. Nucleotide diversity values (π) were generally higher in the protein-coding genes than in the ribosomal DNA regions. When comparing the values across lineages, clade I consistently showed high nucleotide diversity with the greatest values in three out of four loci, followed by clade II. In general, clades III–VI, with more restricted geographic distributions, and hence smaller sample sizes, tended to have lower nucleotide diversity values. Similarly, clade I consistently had the highest number of haplotypes for all loci, followed by clade II, despite the fact that the number of sequences was usually greater in clade II.

Interclade divergence is summarized in Table S1 (Supporting Information). Pairwise divergence values between the clades varied widely. The average number of nucleotide substitution per site (D_{xy}) values ranged from 0.00789 to 0.04639 in ITS, from 0.00889 to 0.07006 in *β-tubulin*, from 0.01789 to 0.10308 in *EF1-α*, and from 0.00324 to 0.01004 in LSU. The *Fst* values measuring the genetic differentiation between clade pairs were always high, between 0.82496 and 1.00000 in ITS, 0.20565 and 1.00000 in *β-tubulin*, 0.63681 and 1.00000 in *EF1-α*, and 0.83686 and 1.00000 in LSU. The number of fixed differences were in the range of 7–26, 0–13, 3–53, and 2–3 in ITS, *β-tubulin*, *EF1-α*, and LSU, respectively. The numbers of polymorphisms shared among clades were low (0 or 1) in all cases, and a varying number of sites polymorphic in one clade but monomorphic in the other were observed.

3.3. Genetic differentiation among populations within phylogenetic species

In clade I, all four geographic groups showed significant *P*-values for Hudson's test, indicating genetic differentiation (Table 3). MDIV indicated no gene flow ($M = 0$) and non-zero divergence

Table 3
Genetic differentiation between pairs of geographic populations according to Hudson's test statistics *KST*, *KS*, and *KT*

Phylogenetic species/intraspecific geographic groups	*KST*	*KS*	*KT*	*P*	*M*	*T*
Clade I						
Eastern North America vs. Mexico	0.402795	1.409091	2.359477	0.000	0	1.3
Eastern North America vs. Alaska	0.523877	0.899621	1.889474	0.000	0	1.5
Eastern vs. Western North America	0.225558	1.569264	2.026316	0.000	NS	NS
Mexico vs. Alaska	0.672072	0.533333	1.626374	0.000	0	2
Mexico vs. Western North America	0.482151	1.604762	3.098901	0.000	0	1.5
Alaska vs. Western North America	0.630542	0.892857	2.416667	0.002	0	1.8
Clade II						
Asia vs. Europe	0.075163	0.651590	0.704545	0.006	NS	NS
Asia vs. Alaska	0.034948	0.611578	0.633725	0.017	NS	NS
Asia vs. Pacific Northwest North America	0.794372	0.411255	2.000000	0.000	0	3
Europe vs. Alaska	0.074096	0.777501	0.839721	0.002	NS	NS
Europe vs. Pacific Northwest North America	0.742424	0.757576	2.941176	0.000	0	4
Alaska vs. Pacific Northwest North America	0.661248	0.635373	1.875630	0.000	0	3.5

Significance was evaluated by performing 1000 permutations for each phylogenetic species clades. Migration (*M*) and divergence time (*T*) between geographic population pairs were estimated using MCMC coalescent simulations in MDIV (see distributions in Figs. S2 and S3).

time (T: 1.3–2.0) between group pairs. The only exception was between the 'Eastern North American' and 'Western North American' groups, where MDIV indicated non-zero levels of gene flow, although the hypothesis of $M = 0$ could not be rejected given the shape of the posterior probability distribution (Fig. S2, Supporting Information).

Geographic groups in clade II also had significant P-values for Hudson's test (Table 3). In most cases, however, MDIV indicated low to intermediate levels of gene flow, although the hypothesis of $M = 0$ could not be rejected. As expected, the 'Pacific Northwest North American' group (clade II/A in Fig. 1) always showed significant results for no gene flow ($M = 0$) and non-zero divergence time (T: 3–4) when compared to other groups (Table 3 and Fig. S3, Supporting Information).

4. Discussion

In recent years, molecular tools have revealed several examples of phylogenetic speciation within complexes that were previously treated as morphological species. The vast majority of these examples dealt with phylogenetic species that were allopatric on a continental scale. For example, morphological species complexes of fungi from the Northern Hemisphere have generally been shown to comprise two major lineages, a Eurasian and a North American (e.g., Shen et al., 2002; Taylor et al., 2006 and the references therein). In most studied fungi, the allopatric phylogenetic clades inhabit similar environments in different continents, which implies a phylogenetic structure that has arisen as a result of the lack of intercontinental dispersal.

On the other hand, while two such major clades (I and II) are also present in *A. muscaria*, we found several other divergent lineages that occupy different habitats or regions in the same continent, sometimes in relatively close proximity. In these cases, spore dispersal is unlikely to be a limiting factor, and adaptation to different ecological niches is a more parsimonious explanation. Such ecoregional diversification is particularly obvious in clade III that is sympatric with the Eurasian clade (II) over the former's entire range, but is predominantly found above treeline (micro-allopatric) in Alaska. Also, clade IV almost exclusively inhabits the mixed pine–oak–hickory forests of the southeastern US and has been collected infrequently as far north as Long Island. Lineages V, VI, and VIII are newly discovered species and have only been found on Santa Cruz Island off the coast of California.

These eight lineages appear to be distinct phylogenetic species with no gene flow among them, as indicated by the results of the PHT test and the tree topologies. Although, in theory, laboratory mating tests may provide information on whether or not *in vitro* interbreeding is possible among different phylogenetic groups, we did not attempt crossing the representatives of the distinct clades to test for increased reproductive isolation with increased genetic distance. Assessments of sporocarp and viable basidiospore production of the 'hybrid' offsprings, the ultimate measure of successful breeding, are virtually impossible to carry out in obligate ectomycorrhizae, such as *A. muscaria*. Nonetheless, the concordance of gene trees fulfills the phylogenetic species recognition criteria following Taylor et al. (2000), and suggest reproductive isolation among the lineages even when multiple lineages occur in sympatry, e.g., in interior Alaska, the central Atlantic coastal region of the US, and on Santa Cruz Island. The non-monophyly of some clades in the *β-tubulin* dataset is likely due to incomplete lineage sorting at that locus. This is particularly plausible, because clades with the largest population sizes (occupying by far the largest geographic areas) were the non-monophyletic ones, while the smaller, regionally endemic clades were monophyletic across all loci. Larger populations require longer periods of time to lose all ancestral alleles.

In addition to the species-level ecoregional endemism, we found evidence for additional phylogeographic structure at the population-level in clades I and II as well. In clade I, coalescent analyses revealed lack of migration and considerable divergence among the four major geographic groups, i.e., 'Alaskan', 'Eastern North American', 'Western North American', and 'Mexican'. The divergence with the weakest support was that found between populations of Eastern vs. Western North America. In this case, the results were only marginally significant, and some current migration could not be ruled out. Future sampling in Canada and the northern Great Plains should provide evidence as to whether Eastern and Western North America represent one or more populations. In clade II, there may be a low to intermediate level of migration among most of the geographic groups spanning Eurasia and Alaska. On the other hand, the 'Pacific Northwest North American' group (clade II/A in Fig. 1) shows unequivocal evidence for both genetic and ecoregional isolation from the rest of clade II. This latter group has only been found in the maritime rainforests from Washington state to southeastern Alaska along the Pacific coast of North America.

Most of the major clades within *A. muscaria* may have diverged as a result of multiple fragmentations and geographic isolation of the ancestral populations due to climatic changes in the late Tertiary and Quaternary. In time, they likely have evolved *in situ* and adapted to the specific plant communities inhabiting different biogeographic regions. Although we cannot estimate the times of divergence of the clades with certainty due to the lack of fossils and the great variance in nucleotide substitution rates in fungi, it seems very likely that the major lineages separated well before the Pleistocene glacial cycles (Geml et al., 2006). On the other hand, the intraspecific phylogeographic groups, shown in Figs. S2 and S3 and in Table 3, may represent groups that became isolated from the rest of their species in the Pleistocene and survived one or more glacial maxima in local refugia.

Our results also point out that while most lineages occupy either entirely allopatric or micro-allopatric habitats, we know very little about the biology and ecology of the different clades that live in true sympatry, i.e., growing within the same forest stand in close proximity. General ecological theory predicts that sympatric taxa will minimize interspecific competition by niche partitioning (Hutchinson, 1957). In the clades under consideration, this may mean host-specificity, inhabiting different soil horizons, and/or favoring sites with different microclimates. Future ecological studies should include representatives of various phylogenetic species to elucidate the ecology of these important mycorrhizal fungi and, in particular, the evolution of host preference.

The implications of our results are not restricted to the *A. muscaria* complex, but are important for biodiversity studies and conservation of ectomycorrhizal taxa in general, and for assessing these fungi's resilience and future responses to climate change. Because mycorrhizal fungi, including *A. muscaria*, play key ecological roles in the decomposition, mineralization, immobilization, and the transfer of nutrients to plants, and because the genetic diversity, distribution, population structure and abundance of particular species are likely to affect the rates and patterns of activity of their communities, knowing and preserving fungal diversity is crucial for sustaining overall functional biodiversity.

Acknowledgments

This research was sponsored by the Metagenomics of Boreal Forest Fungi project (NSF Grant No. 0333308) to D.L.T. and others and the University of Alaska Presidential International Polar Year Postdoctoral Fellowship to J.G. The authors also thank Lisa Grubisha for providing the Santa Cruz Island specimens, and Shawn Houston and James Long at the Biotechnology Computing Research Group at UAF for the technical support in phylogenetic analyses.

Finally, we are grateful to the anonymous reviewers for the helpful comments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2008.04.029.

References

Archie, J.W., 1989. A randomization test for phylogenetic information in systematic data. Syst. Zool. 38, 239–252.

Avise, J.C., 2000. Phylogeography: The History and Formation of Species. Harvard University Press, Cambridge.

Aylor, D.L., Price, E.W., Carbone, I., 2006. SNAP: combine and map modules for multilocus population genetic analysis. Bioinformatics 22, 1399–1401.

Bagley, S.J., Orlovich, D.A., 2004. Genet size and distribution of *Amanita muscaria* in a suburban park, Dunedin, New Zealand. N Z J. Bot. 42, 939–947.

Bowden, L.C., Price, E.W., Carbone, I., 2008. SNAP Clade and Matrix, Version 2. Available from: <http://www.cals.ncsu.edu/plantpath/faculty/carbone/home.html>, Department of Plant Pathology, North Carolina State University.

Faith, D.P., Cranston, P.S., 1991. Could a cladogram this short have arisen by chance alone? On permutation tests for cladistic structure. Cladistics 7, 1–28.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Fu, Y.X., Li, W.H., 1993. Maximum likelihood estimation of population parameters. Genetics 134, 1261–1270.

Geml, J., Davis, D.D., Geiser, D.M., 2005. Phylogenetic analyses reveal deeply divergent species lineages in the genus *Sphaerobolus*. Mol. Phylogenet. Evol. 35, 313–322.

Geml, J., Laursen, G.A., O'Neill, K., Nusbaum, H.C., Taylor, D.L., 2006. Beringian origins and cryptic speciation events in the fly agaric (*Amanita muscaria*). Mol. Ecol. 15, 225–239.

Hudson, R.R., Boos, D.D., Kaplan, N.L., 1992. A statistical test for detecting geographic subdivision. Mol. Biol. Evol. 9, 138–151.

Hutchinson, G.E., 1957. Concluding remarks. Cold Spring Harbor Symp. Quant. Biol. 22, 415–427.

Jenkins, D.T., 1986. *Amanita* of North America. Mad River Press, Eureka.

Johnson, N.C., Gehring, C.A., 2007. Mycorrhizas: symbiotic mediators of rhizosphere and ecosystem processes. In: Cardon, Z.G., Whitbeck, J.L. (Eds.), The Rhizosphere: An Ecological Perspective. Academic Press, Amsterdam, pp. 73–100.

Nei, M., Kumar, S., 2000. Molecular Evolution and Phylogenetics. Oxford University Press.

Nei, M., Li, W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76, 5269–5273.

Nielsen, R., Wakeley, J., 2001. Distinguishing migration from isolation: a Markov chain Monte Carlo approach. Genetics 158, 885–896.

Oda, T., Tanaka, C., Tsuda, M., 2004. Molecular phylogeny and biogeography of the widely distributed *Amanita* species, *A. muscaria* and *A. pantherina*. Mycol. Res. 108, 885–896.

Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Price, E.W., Carbone, I., 2005. SNAP: workbench management tool for evolutionary population genetic analysis. Bioinformatics 21, 402–404.

Read, D.J., Perez-Moreno, J., 2003. Mycorrhizas and nutrient cycling in ecosystems—a journey towards relevance? New Phytol. 157, 475–492.

Rozas, J., Rozas, R., 1999. DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics 15, 174–175.

Shen, Q., Geiser, D.M., Royse, D.J., 2002. Molecular phylogenetic analysis of *Grifola frondosa* (maitake) reveals a species partition separating eastern North American and Asian isolates. Mycologia 94, 472–482.

Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihood with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114–1116.

Swofford, D.L., 2002. PAUP*. Phylogenetic Analysis Using Parsimony (* And Other Methods), 4.0b4a. Sinauer Associates, Sunderland, MA.

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., Fisher, M.C., 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genet. Biol. 31, 21–32.

Taylor, J.W., Turner, E., Townsend, J.P., Dettman, J.R., Jacobson, D., 2006. Eukaryotic microbes, species recognition and the geographic limits of species: examples from the kingdom Fungi. Philos. Trans. R. Soc. Lond. Ser. B 361, 1947–1963.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Trappe, J.M., 1987. Phylogenetic and ecological aspects of mycotrophy in the angiosperms from an evolutionary standpoint. In: Safir, G.R. (Ed.), Ecophysiology of VA Mycorrhizal Plants. CRC Press, Boca Raton, pp. 5–25.

Zwickl, D.J., 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. Dissertation, The University of Texas at Austin.