



Intercontinental divergence in the *Populus*-associated ectomycorrhizal fungus, *Tricholoma populinum*

Lisa C. Grubisha^{1,3}, Nicholas Levsen², Matthew S. Olson^{1,2} and D. Lee Taylor¹

¹Institute of Arctic Biology, 902 N. Koyukuk Drive, 311 Irving 1 Building, University of Alaska Fairbanks, Fairbanks, AK 99775-7000, USA; ²Department of Biological Sciences, Texas Tech University, Lubbock, TX 79409, USA; ³Present address: Biology Program, Centre College, Danville, KY 40422, USA

Summary

Author for correspondence:

Lisa C. Grubisha

Tel: +1 859 238 5370

Email: lisa.grubisha@centre.edu

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- The ectomycorrhizal fungus *Tricholoma populinum* is host-specific with *Populus* species. *T. populinum* has wind-dispersed propagules and may be capable of long-distance dispersal. In this study, we tested the hypothesis of a panmictic population between Scandinavia and North America.

- DNA sequences from five nuclear loci were used to assess phylogeographic structure and nucleotide divergence between continents.

- *Tricholoma populinum* was composed of Scandinavian and North American lineages with complete absence of shared haplotypes and only one shared nucleotide mutation. Divergence of these lineages was estimated at approx. 1.7–1.0 million yr ago (Ma), which occurred after the estimated divergence of host species *Populus tremula* and *Populus balsamifera*/*Populus trichocarpa* at 5 Ma. Phylogeographic structure was not observed within Scandinavian or North American lineages of *T. populinum*.

- Intercontinental divergence appears to have resulted from either allopatric isolation; a recent, rare long-distance dispersal founding event followed by genetic drift; or the response in an obligate mycorrhizal fungus with a narrow host range to contractions and expansion of host distribution during glacial and interglacial episodes within continents. Understanding present genetic variation in populations is important for predicting how obligate symbiotic fungi will adapt to present and future changing climatic conditions.

Introduction

Mycorrhizal symbioses are ubiquitous in nature, with symbiotic mycorrhizal plant species found in c. 90% of all plant families (Trappe, 1987). These symbioses date back over 400 million yr (Redecker *et al.*, 2000), and are integral to ecosystem function, affecting both nutrient cycling and the establishment and growth of plants (Smith & Read, 2008). Despite the importance of the mycorrhizal symbiotic relationship, phylogeographic and taxonomic relationships between host and fungal symbionts remain understudied. The problem is particularly acute for the mycorrhizal fungal partner because morphological traits to discriminate species boundaries and population structure are often limited.

For many years, the ubiquitous dispersal hypothesis (Finlay, 2002) has been accepted by many researchers. This hypothesis contends that microbes are capable of long-distance dispersal owing to their small size and the large number of propagules (Finlay, 2002), and therefore population structure across vast geographic areas with similar habitat should be minimal. This principle is supported by global panmictic population structure in some fungi, including several human pathogens (Pringle *et al.*, 2005; Rydholm *et al.*, 2006) and root endophytes (Queloz *et al.*,

2011). Other studies, however, have come to different conclusions, with several examples in the fungal literature of morphologically similar isolates traditionally placed in the same species that, in fact, have deep phylogenetic and phylogeographic structure when analyzed with molecular methods (Taylor *et al.*, 2006; Douhan *et al.*, 2011). Even at fine spatial scales, ectomycorrhizal fungi have been shown to have phylogenetic structure resulting in sympatric cryptic species as in *Rhizopogon* (Kretzer *et al.*, 2003), *Cenococcum* (Douhan & Rizzo, 2005) and the *Amanita muscaria* complex (Geml *et al.*, 2006, 2008).

Intercontinental geographic distribution for any species may result from vicariance or long-distance dispersal. Intercontinental vicariance is, by definition, ancient, but long-distance dispersal may have occurred at any point since continental separation. Thus, recent long-distance dispersal and vicariance should have very different genetic signatures, while ancient long-distance dispersal may be difficult to distinguish from vicariance. In the case of mycorrhizal fungi that are obligate symbionts, suitable host species need to be present for intercontinental gene flow to be successful, a characteristic that is more constraining for fungal species with a narrow host range. Studies of some ectomycorrhizal fungi with widespread distributions have revealed

distinct phylogenetic lineages with biogeographic and host boundaries, for example, *Pisolithus* (Martin *et al.*, 2002), *Auritella* (Matheny & Bougher, 2006), *A. muscaria* (Geml *et al.*, 2006, 2008) and *Tylopilus ballouii* (Halling *et al.*, 2008). Although a large number of ectomycorrhizal fungi have Holarctic distributions (Tedersoo *et al.*, 2010), there are too few studies of genetic variation from species with a Holarctic biogeographic range to elucidate general trends (Douhan *et al.*, 2011).

Tricholoma populinum Lange (Basidiomycota, Agaricales) is a mushroom-forming ectomycorrhizal fungus that is host-specific with species in the genus *Populus* (Salicaceae), whereas *Populus* associate with many ectomycorrhizal fungi (Cripps & Miller, 1993; Krpata *et al.*, 2008; Bahram *et al.*, 2010). *Populus* (poplars, cottonwoods, aspens) is composed of *c.* 29 upland and riparian species with largely Northern Hemisphere distributions (Eckenwalder, 1996). *Populus* species have either a Palearctic or Nearctic distribution; however, no individual *Populus* species has a native Holarctic range (Dickmann & Kuzovkina, 2008). The European poplars comprise a polyphyletic group of three to four species (Hamzeh & Dayanandan, 2004; Cervera *et al.*, 2005), whereas 12 species are found in North America (Eckenwalder, 1996). Except for *Populus tremula* L. (European aspen), which is sister to the North American *Populus tremuloides* Michx. (quaking aspen), the European poplar species are most closely related to Asian species (Cervera *et al.*, 2005). The most well-resolved phylogenetic reconstructions of the entire genus are based on amplified fragment length polymorphism (AFLP) and intersimple sequence repeat (ISSR) markers for which phylogenetic accuracy above the species-level is low (Simmons *et al.*, 2007; García-Pereira *et al.*, 2010) and models of evolution are not well developed (Ehrich *et al.*, 2009). Divergence dates have been estimated for only the basal node (Tuskan *et al.*, 2006), based on fossil data, and the very recent split (*c.* 75 000 yr ago) between *Populus trichocarpa* and *Populus balsamifera* in North America (Levens *et al.*, 2012). *T. populinum* co-occurs with *P. tremula*, *Populus nigra*, and *Populus alba*, in Europe and with *P. balsamifera* L. (balsam poplar) and *P. trichocarpa* Torr. & Gray (black cottonwood) in North America, but it is not known whether this species comprises a panmictic intercontinental fungal population or if there are separate fungal lineages that have diverged as a result of allopatric isolation or the evolution of host specificity. In this paper, we used analyses of five nuclear loci to test the hypothesis that *T. populinum* constitutes a single panmictic population through examination of genetic variation of *T. populinum* populations from Scandinavia and North America that are associated with different *Populus* host species, *P. tremula* in Scandinavia and *P. balsamifera* and *P. trichocarpa* in North America.

Materials and Methods

Study area and taxon sampling

Specimens used in this study are detailed in Supporting Information, Table S1, including name, collection location, host, and GenBank accession numbers for each locus sequenced.

Collections were from herbaria or collected for this project. A total of 48 *T. populinum* collections were used in this study: 36 from North America and 12 from Norway and Sweden. In North America, *T. populinum* was collected associated with two hosts, *P. balsamifera* and *P. trichocarpa*. Host association could not be verified from herbaria collections from Colorado, Michigan and Ontario. In Norway and Sweden, the collections were associated with *P. tremula*. The maximum distance within North America between host populations of *P. balsamifera* (interior Alaska) and *P. trichocarpa* (Pacific Northwest, USA) was approx. 2500 km (Table S1, Fig. 1). The maximum distance between Scandinavian populations was approx. 400 km.

DNA Isolation, PCR amplification, and sequencing

Dried fungal tissue from the pileus (mushroom cap) or stipe (stalk) was homogenized in a bead beater. DNA was isolated using either Qiagen DNeasy Plant Mini Kit (Qiagen) or E-Z 96® Fungal DNA Kit (Omega Bio-tek, Norcross, Georgia, USA) following the manufacturer's instructions.

In this study six loci were sequenced that included one mitochondrial locus, the sixth subunit of the adenosine triphosphatase gene (*ATP6*); and five nuclear loci: the nuclear ribosomal internal transcribed spacer region (ITS) that included both ITS1 and ITS2 plus the 5.8S ribosomal subunit gene, the largest subunit of the RNA polymerase II binding protein (*RPB1*), the second largest subunit of the RNA polymerase II binding protein (*RPB2*), translation elongation factor 1- α gene (*TEF1- α*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*).

Loci were PCR-amplified in a 20 μ l final volume of the following mixture: 0.05 U μ l⁻¹ JumpStart™ REDTaq™ DNA polymerase (Sigma), 1 $\times$ PCR buffer (100 mM Tris-HCL (pH 8.3 at 25°C), 500 mM KCL, 15 mM MgCl₂, 0.01% (w/v) gelatin), 0.3 μ M of each primer, 2.5 mM MgCl₂, 0.2 mM dNTPs, and 2.0 μ l of 1 : 10 diluted genomic DNA. Initial PCR amplification used previously published primers and thermocycler protocols (Table 1). New primers were designed using Primer3 (Rozen & Skaletsky, 2000) for *RPB1*, *RPB2*, and *GAPDH* (Table 1). Thermocycler (PTC-220 thermocycler, Programmable Thermal Controller) parameters for amplification of the ITS region were 95°C for 5' min, 35 cycles of 94°C for 30's, 54°C for 40's, and 72°C for 60's, followed by a 10' min final extension at 72°C. PCR conditions for *ATP6* followed Kretzer & Bruns (1999). Using the new primers (Table 1), PCR amplification was performed using a touchdown program: 95°C for 5' min, 10 cycles of 94°C for 30's, 68°C for 30 s, -1.0°C per cycle, 72°C for 1.5 min, then 29 cycles of 94°C for 30 s, 58°C for 30's, 72°C for 1.5 min, and a final extension of 72°C for 10 min for *GAPDH*. The touchdown annealing temperatures were decreased to 65 and 55°C for the new sets of primers for both *RPB1* and *RPB2*. PCR amplicons were electrophoresed in a 1.5% agarose gel, and visualized by staining with ethidium bromide.

Polymerase chain reaction amplification products were cleaned using ExoSAP-IT (US Biochemical Corp., Cleveland, OH, USA). Cycle-sequencing of purified PCR products was performed using the same primers as for PCR amplification and

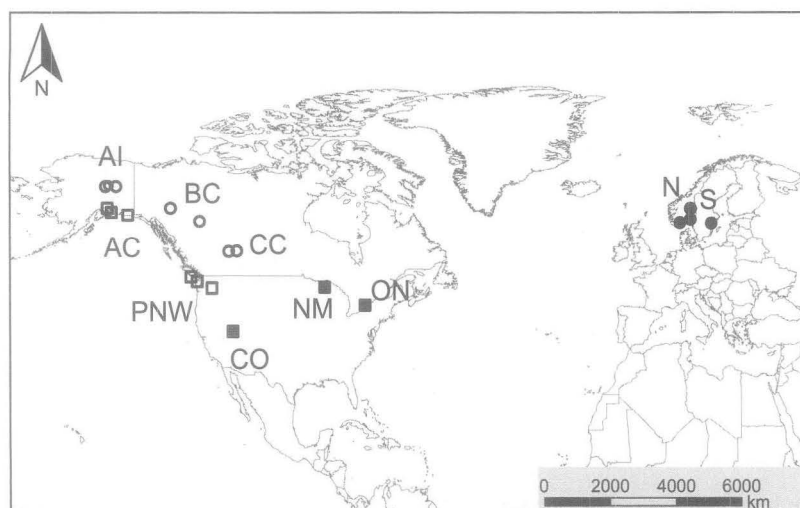


Fig. 1 Approximate collection locations for *Tricholoma populinum* isolates used in this study. The maximum distance of 2500 km from the Pacific Northwest (PNW) to Alaska Interior (AI) encompassed collecting sites from *Populus balsamifera* and *Populus trichocarpa* hosts. Sites from herbarium collections in Norway and Sweden represent a maximum distance of approx. 400 km from Uppsala, Sweden, to Oslo, Norway. AI, Alaska Interior, USA; AC, Alaska Coast, USA; BC, British Columbia, Canada; CC, Central Canada; PNW, Pacific Northwest, Canada and USA; CO, Colorado, USA; NM, Northern Michigan, USA; ON, Ontario, Canada; S, Sweden; N, Norway. Symbols indicate which *Populus* host species was present: closed circles, *P. tremula*; open circles, *P. balsamifera*; open squares, *P. trichocarpa*; closed squares, *P. balsamifera* may be found in these areas, but the exact *Populus* host species is not known.

Table 1 Primers used in this study

Locus	Primer name	Nucleotide sequence (5'–3')	Reference
ITS	ITS1F	CTTGGTCATTAGAGGAAGTAA	Gardes & Bruns (1993)
ITS	ITS4	TCCTCCGCTTATTGATATGC	White <i>et al.</i> (1990)
<i>TEF1-α</i>	EF1-983F	GCYCCYGGHCAYCGTGAYTTYAT	Rehner & Buckley (2005)
<i>TEF1-α</i>	EF1-1953R	CCRGCRACRGTRTGCTCAT	http://aftol1.biology.duke.edu/pub/primers/viewPrimers
<i>ATP6</i>	ATP6-3f	TCTCCTTTAGAACAATTTGA	Kretzer & Bruns (1999)
<i>ATP6</i>	ATP6-6r	AACTAATARAGGAAGCTAAAGCTA	M. Binder (unpublished; http://www.clarku.edu/faculty/dhibbett/Protocols_Folder/Primers/Primers.htm)
<i>RPB1</i>	gRPB1-A forward	GA(G/T)TGTC(T/G)GG(A/T)CATTTTGG	Stiller & Hall (1997)
<i>RPB1</i>	aRPB1-B reverse	TCCGC(A/G)CC(C/T)TCTTC(C/T)TTGG	Matheny <i>et al.</i> (2002)
<i>RPB1</i>	RPB1tp1f	CCATCCTGGTACGCGCATTTTC	This study
<i>RPB1</i>	RPB1tp1r	TCC GCG CCT TCT TCT TTG G	This study
<i>RPB2</i>	bRPB2-6f	TGGGGYATGGTNTGYCCYGC	Matheny (2005)
<i>RPB2</i>	bRPB2-7.1R	CCCATRGCTGYTTMCCCATDGC	Matheny (2005)
<i>RPB2</i>	RPB2tp1f	AGGTCAAGCGTGGGTCTC	This study
<i>RPB2</i>	RPB2tp1r	TGAGGAGACTATTGGGAGATG	This study
<i>GAPDH</i>	GPD1F	CGGCCGTATCGTCTCCGTAATGC	den Bakker <i>et al.</i> (2004)
<i>GAPDH</i>	GPD2R	GAGTA(AT)CC(GC)CATTCGTTATCGTACC	den Bakker <i>et al.</i> (2004)
<i>GAPDH</i>	GPD1tp1f	ATGCCTACAGCCCTTTTCATC	This study
<i>GAPDH</i>	GPD1tp1r	GCACAACCAAGTCCACGA	This study

ITS, nuclear ribosomal internal transcribed spacer region (ITS1 and ITS2 plus the 5.8S ribosomal subunit gene); *TEF1- α* , translation elongation factor 1- α gene; *ATP6*, the sixth subunit of the adenosine triphosphatase gene; *RPB1*, the largest subunit of the RNA polymerase II binding protein; *RPB2*, the second largest subunit of the RNA polymerase II binding protein; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

Applied Biosystems (ABI, Carlsbad, California, USA) BigDye[®] version 3.1 Terminator Kit. Samples were run on an ABI 3130 automated capillary DNA sequencer at the Core Facility for Nucleic Acid Analysis in the Institute of Arctic Biology, University of Alaska Fairbanks. ITS sequences from 19 North American isolates used in this study were generated by the MIT Broad Institute as part of a large-scale fungal biodiversity project of D. Lee Taylor. Sequences were assembled into contigs and edited using the program codoncode aligner version 1.52 (CodonCode Inc., Dedham, MA, USA). Coding regions and introns were

identified using the web server AUGUSTUS version 2.4 (Stanke *et al.*, 2008). Sequences were deposited in GenBank as accessions JN019383–JN019533 and JN019584–JN019753 (Table 1). Only one haplotype was identified for *ATP6* for all *T. populinum* isolates and thus this locus was not included in further analyses.

Haplotype reconstruction

Haplotypes of heterozygous sequences were determined using three methods. Analysis of chromatograms revealed heterozygous

multiple base insertion/deletions (indel) or more than one heterozygous indel in three ITS sequences. PCR products for these ITS sequences were cloned using the TOPO-TA cloning kit (Invitrogen) following manufacturer's instructions and sequenced. For other loci with single heterozygous indel sites, chromatograms were of high quality with clear single peaks until the point of the indel and then double peaks were present for the remainder of the sequence. These sequences were subtracted and a gap added to the appropriate sequence, resulting in two haplotypes following Peters *et al.* (2007). Finally, for heterozygous sequences with single nucleotide substitutions, haplotypes were reconstructed from analyses in a Bayesian framework using the program phase version 2.1.1 (Stephens *et al.*, 2001; Stephens & Donnelly, 2003). To check for consistency of results, haplotype frequency estimates and goodness-of-fit measures from five independent runs were compared using the parameters: 10 000 iterations, 100 thinning intervals, and a burn-in of 10 000 generations. Haplotype pairs with a posterior probability of ≥ 0.90 were accepted and used for haplotype analyses.

DNA polymorphism and nucleotide divergence

Estimates of nucleotide polymorphism were conducted using DNASP version 5.10 (Librado & Rozas, 2009). Alignment gaps were removed for all analyses. The number of haplotypes, h , segregating sites, S , Watterson's θ (an estimate of the population mutation rate, $\theta = 4N_e\mu$, where N_e is the effective population size and μ is the mutation rate per generation (Watterson, 1975)), and average nucleotide diversity, π (Nei, 1987), were calculated to determine the degrees of DNA polymorphism in Scandinavia vs North America and across hosts.

Estimates of nucleotide divergence were conducted using DNASP (Librado & Rozas, 2009). Nucleotide divergence between populations based on hosts and continents was estimated as the number of fixed nucleotide differences between populations, the number of polymorphic mutations that occur in one population but are monomorphic in the second, the number of shared mutations, the average number of nucleotide differences between populations, K , nucleotide divergence (average number of nucleotide substitutions per site between populations), D_{xy} , and the number of net nucleotide substitutions per site between populations, D_a .

Population differentiation

Population structure was identified using the Bayesian clustering method implemented in Structure v2.3.3 (Pritchard *et al.*, 2000; Hubisz *et al.*, 2009). Haplotypes were allowed to have mixed ancestry in each of the K clusters (admixture model; Pritchard *et al.*, 2000; Hubisz *et al.*, 2009). To assess within continent genetic structure, a prior was set for each individual's sampling location (locprior model) that detects weak population structure (Hubisz *et al.*, 2009). Markov chain Monte Carlo (MCMC) simulations were run for $K = 1$ to $K = 10$ genetic partitions, with 15 replicate runs for each K . For each run a burn-in of 20 000

MCMC iterations was followed by 80 000 MCMC iterations. The number of genetic partitions was determined by estimating the ad-hoc statistic ΔK , which is the largest second-order rate of change in the likelihood distribution between successive runs of K (Evanno *et al.*, 2005), using Structure harvester v0.6 (available at http://taylor0.biology.ucla.edu/struct_harvest/).

Phylogenetic analysis

FASTA files of nonphased sequences were compiled into multiple sequence alignments using the program Opal of the Opalescent version 1.0 (Wheeler & Kececioglu, 2007) package within Mesquite version 2.73 (Maddison & Maddison, 2010) and manually edited. For ITS sequences that had two alleles as a result of indels, a consensus sequence was determined for each isolate by deleting indels that represented intra-isolate allelic polymorphism. GenBank sequences of *Tricholoma portentosum* and *Tricholoma myomyces* were added as outgroups for *TEF1- α* (EF421084, DQ367429), ITS (AF357015, DQ825428), *RPB1* (EF421047, DQ842013) and *RPB2* (EF421014, DQ367436). *Tricholoma scalpturatum* (GU060260) and *Tricholoma argyraceum* (GU060239) were outgroups for *GAPDH*. Alignments were deposited on TreeBASE (Accession 12241). Bayesian analyses were conducted to estimate gene genealogies using MrBayes v. 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Nucleotide substitution models for each locus were estimated by employing the Akaike Information Criterion (AIC; Akaike, 1974) using MrModeltest v. 2.3 (Nylander, 2004) and PAUP* (Swofford, 2003). The GTR model of DNA substitution + gamma (Γ) was employed for *TEF1- α* , *RPB2*, ITS, *GAPDH* and GTR + invariant (I) for *RPB1*. Gamma distribution was approximated using four rate categories. Gaps were treated as missing data. Two runs of 10 000 000 generations were performed, sampling every 1000 generations and saving 10 000 trees. The first 2500 genealogies were discarded as burn-in. To determine if a stationary phase was reached at this burn-in, scatterplots were generated. Convergence for each run was also assessed by plotting the log-likelihood values against generation time using Tracer 1.5 (available at: <http://tree.bio.ed.ac.uk/software/tracer/>). Posterior probabilities were determined from the remaining 7500 trees and a 50% majority rule consensus tree was computed and viewed in FigTree ver.1.3.1 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Divergence time estimates for *Tricholoma*

A Bayesian MCMC method for multispecies coalescent analyses was implemented in *Beast ver. 1.6.1 (Drummond *et al.*, 2006; Drummond & Rambaut, 2007; Heled & Drummond, 2010) for *T. populinum* North America associated with *P. balsamifera* and *T. populinum* Scandinavia associated with *P. tremula*. One advantage of using *Beast is that the standard error decreases with increased sample size for the species analyzed (Heled & Drummond, 2010). We had 17 *T. populinum* isolates from the North American *P. balsamifera* host and 12 *T. populinum* isolates from the Scandinavian *P. tremula* host. The three isolates

that formed a monophyletic clade in all single gene phylogenies (O-F170922, O-F220341, AT2005140) were included as the outgroup. Closely related *Tricholoma* species included in the single gene genealogies, for example, *T. aurantium*, *T. spp.* DAVFP26750, were not included in *Beast analyses because the minimum requirement for estimation of population size is at least two isolates per species (Heled & Drummond, 2010). An assumption of *Beast is that sequences are free of recombination. The largest nonrecombining block of DNA sequence for each locus was identified using the online server IMGc (Woerner *et al.*, 2007).

Nexus files for each alignment of phased, recombination-free haplotypes of five loci (*TEF1- α* , *GAPDH*, *RPB1*, *RPB2* and ITS) were imported separately into BEAUti v1.6.1. Alignment gaps were deleted. Loci were unlinked for nucleotide substitution model, molecular clock model and tree model. The Hasegawa, Kishino and Yano (HKY) model of nucleotide substitution was used for each locus. The molecular clock hypothesis was tested for each locus and could not be rejected using the relative rate test of Tajima (1993) employed in Mega v. 4.0 (Tamura *et al.*, 2007). Therefore, a strict molecular clock was set for each locus. A substitution rate was set for the ITS of 1.4×10^{-9} substitutions per site yr^{-1} (Kasuga *et al.*, 2002) and the relative rates for the other loci were estimated in Beast. This method was chosen instead of fixing all loci to the same rate because that method has been shown to substantially decrease the estimate of species divergence (Heled & Drummond, 2010). Analyses were also conducted using the lower and upper ranges for the estimated substitution rates of 0.1×10^{-9} and 2.7×10^{-9} for ITS (Kasuga *et al.*, 2002). The tree model parameters were a random starting tree for each locus, with the species tree prior set to a Yule process. BEAUti v1.6.1 was used to create Beast XML files. Short preliminary runs were performed to check operators for optimal performance that were adjusted in the final runs. To assess consistency across runs, two independent runs were performed, each with 20 000 000 generations, sampling every 2000 generations. Log files for each run were viewed in Tracer ver. 1.5 to verify that effective sample size (ESS) values were > 200 and that parameters had reached a stationary stage after a 10% burn-in. The two species tree files (10 000 trees each) were combined using LogCombiner ver. 1.6.1 after discarding a burn-in of 2500 trees from each run. The LogCombined tree file of 15 000 trees was imported into TreeAnnotator ver. 1.6.1. A summary tree was produced and viewed in FigTree.

A second method of estimating divergence time employed a calibration based on divergence of Ustilaginomycotina and Agaricomycotina using Beast v. 1.6.1 (Drummond *et al.*, 2006; Drummond & Rambaut, 2007). Because of the lack of fossil evidence for *Tricholoma*, a prior for the time to the most recent common ancestor (tMRCA) with a normal distribution was set between *Ustilago* (Ustilaginomycotina) and Agaricomycotina at 430 (± 50) million yr ago (Ma) based on Berbee & Taylor (2001). Nexus files of sequence alignments of *RPB2* (707 bp) and the 5' end of the nuclear large subunit ribosomal RNA gene (nLSU; 513 bp) for a 12 taxon data set were imported into BEAUti ver. 1.6.1 to produce XML files for input in BEAST.

The taxa chosen followed Geml *et al.* (2004) and Matheny *et al.* (2009) and are listed here including GenBank accession numbers for nLSU and *RPB2*, respectively: *Endocronartium harnessii* (AY700193, DQ234551), *Ustilago tritici* (DQ094784, DQ846896), *Auricularia* sp. (AY634277, DQ366278), *Hygrophoropsis aurantiaca* (AY684156, AY786059), *Marasmius alliaceus* (AY635776, AY786060), *Laccaria ochropurpurea* (AY700200, DQ472731), *Alnicola escharoides* (AY380405, AY337411), *T. portentosum* (U76464, EF421014), *Tricholoma myomyces* (U76459, DQ367436), *Tricholoma* spp. AT2005140 (JN019647, JN019704), *T. populinum* O-F70009 (Norway) (JN019648, JN019710), *T. populinum* LCG2003 (Alaska) (JN019649, JN019720). Nucleotide substitution model and molecular clock model were unlinked for the two loci. The GTR + Γ nucleotide substitution model with four rate categories was employed for *RPB2* and the Tn93 + Γ model for nLSU as estimated by jModeltest v. 0.1.1 (Guindon & Gascuel, 2003; Posada, 2008). An uncorrelated lognormal relaxed molecular clock (Drummond *et al.*, 2006) was employed with the tree prior set to a Yule process using a randomly generated starting tree. Prior constraints on monophyletic clades were based on previously published studies (Matheny *et al.*, 2006, 2009). *Tricholoma* was constrained to be a monophyletic group, with no further constraints within the genus except that *T. populinum* Norway and Alaska were constrained to be monophyletic. The analysis was run for 10 000 000 generations, sampling every 1000 trees for each of two replicates to assess consistency among runs. Results were assessed as described earlier.

Divergence time estimates for *Populus*

*Beast analyses of *Populus* host species included 48 phased haplotypes (*P. balsamifera*, 22; *P. trichocarpa*, 20; *P. tremula*, six) of five nuclear loci (195487, 230673, 560714, 751129, and 816152; Levens *et al.*, 2012). All loci adhered to molecular clock assumptions and were set as unlinked for nucleotide substitution model, molecular clock model, and tree model. Nucleotide substitution models for each locus were determined using jModeltest v. 0.1.1, to be as follows: 195487, HKY; 230673, GTR + I; 560714, HKY; 751129, GTR + I; 816152, GTR. The strict molecular clock model was employed for each locus with mean rate of 2.5×10^{-9} substitutions per site yr^{-1} (Tuskan *et al.*, 2006). Each locus had a random starting tree generated under a Yule process. We performed two independent *Beast runs of 100 000 000 generations each with a 10 000 generation sampling interval. Gene sequences for *P. trichocarpa*, *P. balsamifera*, and *P. tremula* are available on GenBank from previous studies (Keller *et al.*, 2010; Levens *et al.*, 2012).

Results

Identification of isolates

Six herbarium collections of *T. populinum* were misidentified. The results of Blast searches of the ITS sequences for these collections is presented in Table S2.

Haplotype reconstruction

Haplotypes determined by Phase for *T. populinum* sequences from *TEF1-α*, ITS, *RPB1*, and *RPB2* all had posterior probabilities (PPs) > 0.90. Eight *GAPDH* sequences from North American collections (LCG2191, LCG2213, LCG2246, LCG2088, LCG2074, LCG2050, LCG2056, LCG2065) had PPs < 0.90 and were not included in analyses requiring phased haplotypes. PCR errors were not observed in the three ITS sequences that were cloned.

Haplotype and nucleotide diversity and divergence

Aligned *T. populinum* sequence lengths, with gaps, for loci *GAPDH*, *RPB1*, *RPB2* and *TEF1-α* were 829, 715, 628, and 800 bp, respectively. The ITS alignment consisted of 588 bp when indels that represented intra-isolate allelic polymorphism were included as phased haplotypes. Colorado isolate LCG2307 had one allele with a single 10 bp deletion in the ITS2 region but was otherwise identical to the second allele. Intra-isolate indel ITS variation was not present in the Scandinavian isolates.

Tricholoma populinum haplotypic diversity was similar across populations and hosts (average Scandinavia $H_d = 0.487$; average North American $H_d = 0.479$ Table 2). Haplotypes were not shared between Scandinavia and North America; in contrast, haplotypes were shared among North American populations for all loci. Average nucleotide diversity (π) varied from a high of 0.00254 in the *GAPDH* locus in *T. populinum* North America population to a low of 0.00014 in ITS of the *T. populinum* host *P. trichocarpa* population (Table 2).

In all six *T. populinum* nuclear loci, only one nucleotide position in *RPB1* was polymorphic in both Scandinavian and North American regions (Table 3). The remaining polymorphic sites were either fixed for differences between Scandinavia and North America, or polymorphic in one region and monomorphic in the other (Table 3). By contrast, comparisons of North American *T. populinum* populations collected near different host species shared polymorphic sites (range of one to six shared mutations) and no fixed differences (Table 3). Higher pairwise nucleotide divergence was found between Scandinavian *T. populinum* populations and each *T. populinum* population within *Populus* hosts in North America (Scandinavian to North American average $K = 8.1888$, average $D_{xy} = 0.01152$, average $D_a = 0.01016$; Table 3) than among North American *T. populinum* populations with two *Populus* hosts for K (average $K = 1.0170$), D_{xy} (average $D_{xy} = 0.00138$), and D_a (average $D_a = 0.00004$) (Table 3).

Population structure

The population structure of *T. populinum* within and among Scandinavian and North American regions and between *Populus* host species was assessed with the program Structure (Pritchard *et al.*, 2000). Two clusters were identified ($K = 2$) based on the ad hoc statistic ΔK of Evanno *et al.* (2005) ($\Delta K = 326$) that align perfectly with Scandinavian and North American regions and *Populus* host species (*P. tremula* vs *P. trichocarpa*/*P. balsamifera*),

with no admixture (Fig. S1). Structure results did not reveal genetic clusters within regions or hosts within North America.

Phylogenetic analyses

Bayesian inference of the *T. populinum* phylogenetic tree using our five nuclear loci provided strong support (PP = 1.0, four phylogenies) for monophyly of Scandinavian and North American populations of *T. populinum* (Fig. 2). Isolates O-F170922, O-F220341 and AT2005140 formed a strongly supported clade (PP = 1.0, four phylogenies) outside of *T. populinum* for each gene phylogeny. Isolate DAVFP26750 was the sister taxon to *T. populinum* in all gene phylogenies.

Divergence time estimates for *Tricholoma*

The largest recombination-free sequence blocks were used in *Beast analyses: *TEF1-α* was truncated to 1–675 bp, *RPB1* to 1–589 bp, *RPB2* 1–561 bp with deletion of phased haplotypes O-F70600a, O-F70600b, O-F51644b, O-F51642b, LCG2088b, LCG2074b, and for *GAPDH* the entire sequence was used after phased haplotypes AI02a and PC05b were removed. Recombination was not detected in ITS sequences. *Beast analyses resulted in a PP of 1.0 at both nodes and recovered *T. populinum* North America and Scandinavia as sister lineages (Fig. S2). The mean estimate of the divergence time between Scandinavia (*P. tremula* host) and North America (*P. balsamifera* host) was 1.7 Ma (95% highest posterior density (HPD): 0.76, 2.95 Ma). Estimates based initially on the upper and lower estimates of mutation rate for ITS from Kasuga *et al.* (2002) resulted in 0.87 Ma (95% HPD: 0.35, 1.46) and 23.55 Ma (95% HPD: 9.77, 41.98), respectively.

An estimate of divergence using a calibration point of divergence of the Ustilaginomycotina and Agaricomycotina resulted in an estimated divergence between North American and Scandinavian *T. populinum* at approx. 1.0 Ma (95% HPD: 0.17, 2.40) (Fig. 3). All nodes show evidence of strong support based on a PP of 0.98–1.0 (Fig. 3).

Divergence time estimates for *Populus*

Populus tremula and *P. balsamifera*/*P. trichocarpa* clades were recovered in *Beast with a PP of 1.0 (Fig. S3). The mean estimate of divergence time between *P. tremula* and *P. balsamifera*/*P. trichocarpa* was 5.04 Ma (95% HPD: 3.24–6.84 Ma).

Discussion

Intercontinental lineage divergence

Our results strongly support a hypothesis of lineage divergence and a lack of contemporary gene flow between North American and Scandinavian populations of *T. populinum*. Migration corridors were present either via the Bering Land Bridge (BLB) from eastern Siberia to Alaska or via the North Atlantic Land Bridge (NALB) between Europe and North America through

Table 2 Nucleotide polymorphism for populations of *Tricholoma populinum* identified by host and geographic location

Host	Location and locus	<i>n</i>	<i>h</i>	<i>S</i>	<i>H_d</i>	θ _W /site	π
<i>Populus tremula</i>	Norway/Sweden						
	GAPDH	24	3	2	0.424	0.00065	0.00055
	RPB1	24	4	3	0.649	0.00112	0.00109
	RPB2	24	7	6	0.851	0.00256	0.0037
	TEF1-α	18	2	1	0.209	0.00036	0.00026
	ITS	24	3	2	0.301	0.00092	0.00053
	Mean				0.487	0.001	0.001226
<i>Populus balsamifera</i> , <i>Populus trichocarpa</i> , <i>Populus</i> spp. ^a	North America						
	GAPDH	54	13	11	0.855	0.00291	0.00254
	RPB1	70	6	8	0.391	0.00232	0.00193
	RPB2	70	6	7	0.489	0.00231	0.00179
	TEF1-α	64	6	4	0.52	0.00106	0.00105
	ITS	70	4	3	0.138	0.00108	0.00025
	Mean				0.479	0.002	0.002
<i>Populus balsamifera</i>	North America						
	GAPDH	20	8	7	0.847	0.00238	0.00235
	RPB1	34	4	8	0.365	0.00274	0.00182
	RPB2	34	4	6	0.273	0.00234	0.00133
	TEF1-α	34	4	3	0.544	0.00092	0.0011
	ITS	34	3	2	0.169	0.00084	0.0003
	Mean				0.440	0.002	0.001
<i>Populus trichocarpa</i>	North America						
	GAPDH	22	6	8	0.831	0.00265	0.00246
	RPB1	24	3	6	0.304	0.00225	0.00134
	RPB2	24	4	4	0.609	0.00171	0.00198
	TEF1-α	24	4	3	0.308	0.001	0.00059
	ITS	24	2	1	0.083	0.00046	0.00014
	Mean				0.427	0.002	0.001

^aFor analyses of the North American host *P. balsamifera*, *P. trichocarpa* and *Populus* spp. category, isolate TRTC150613 and isolates from Colorado from the unverified *Populus* host were included.

n, number of phased haploid sequences; *h*, number of haplotypes; *S*, number of segregating sites; *H_d*, haplotypic diversity; θ_W, Watterson's *theta*; π, average pairwise nucleotide diversity.

Greenland. The NALB was intact until *c.* 40 Ma, but functional as late as 25–15 Ma (Milne, 2006). However, recently, Denk *et al.* (2010) concluded that the NALB may have been an active route into the late Miocene (11.6–5.3 Ma). The BLB was severed approx. 5.4–5.5 Ma (Gladenkov *et al.*, 2002) and may have been functional continuously between 65 and 5.4 Ma, (Milne, 2006). Ingvarsson (2005), using an average (five gene) silent site divergence between *P. tremula* and *P. trichocarpa* of 6.1% and assuming a silent site substitution rate of 5.0–8.0 × 10^{−9}, estimated the divergence of *P. tremula* and *P. trichocarpa* to have been between 3.8 and 6.2 Ma. Our coalescent-based estimates also place the divergence of *P. tremula* and *P. balsamifera*/*P. trichocarpa* during the Late Miocene to Pliocene (5.3–2.6 Ma) epochs, roughly coincident with the opening of the Bering Strait (Milne, 2006). However, estimates of divergence in *Tricholoma* are much more recent and place the divergence of these lineages during the Pleistocene (2.6–0.01 Ma) epoch. Our divergence estimates strongly suggest that *Tricholoma* and its *Populus* hosts did not undergo intercontinental migration in tandem.

Possible explanations for the strongly supported, but more recent, intercontinental lineage divergence within *T. populinum* include the following: allopatric isolation of *T. populinum* populations in Scandinavia and North America that was coincident with speciation of *Populus* hosts (*P. tremula* and *P. balsamifera*

/*P. trichocarpa*); effects of population contraction and expansion during the glacial and interglacial cycles of the Pleistocene; and founder effect followed by genetic drift after a single, rare long-distance dispersal event. Since there were no shared haplotypes between continents, analyses of directional migration were not possible.

Determining the possible role coevolution with different *Populus* species (*P. tremula* vs *P. balsamifera*/*P. trichocarpa*) had on intercontinental *T. populinum* divergence will require additional sampling of host populations within Europe and North America or reciprocal inoculation trials. Based on the taxonomic distribution of the host species of *T. populinum*, it is unlikely that the North American and European lineages of *T. populinum* diverged solely as a result of cospeciation with *Populus*. It is curious that there are few collections of *T. populinum* in North America with poplar species other than *P. trichocarpa* and *P. balsamifera*, because *P. tremula* and *P. trichocarpa*/*P. balsamifera* reside in two different and well-diverged sections of the genus. This enigma suggests that environmental factors associated with boreal forest-like environments may limit the distribution of *T. populinum*.

Overall, the extent to which *T. populinum* occurs in Europe, Asia and North America is not well documented. *T. populinum* is known to occur with *P. alba* and *P. nigra* and these populations

Table 3 Nucleotide divergence between *Tricholoma populinum* populations from Scandinavia and North America regions and by host

Region or host and locus	No. of polymorphic sites	No. of fixed differences	P1 (continent or host)	P2 (continent or host)	Shared mutations	K	Dxy	Da
Scandinavia vs North America ^a			Scandinavia	North America				
GAPDH	21	8	2	11	0	9.95400	0.01201	0.01046
RPB1	11	1	2	7	1	4.29400	0.00601	0.00449
RPB2	13	0	6	7	0	4.94600	0.00788	0.00513
ITS	12	8	1	3	0	9.13900	0.01595	0.01563
TEF1- α	17	12	1	4	0	12.61100	0.01576	0.01511
Mean						8.18880	0.01152	0.01016
<i>Populus tremula</i> vs <i>Populus balsamifera</i>			<i>P. tremula</i>	<i>P. balsamifera</i>				
GAPDH	17	8	2	7	0	9.85	0.01188	0.01043
RPB1	11	1	2	7	1	4.24500	0.00594	0.00448
RPB2	12	0	6	6	0	4.84600	0.00772	0.00520
ITS	12	8	2	2	0	9.19600	0.01577	0.01536
TEF1- α	16	12	1	3	0	12.67000	0.01584	0.01516
Mean						8.16140	0.01143	0.01013
<i>P. tremula</i> vs <i>Populus trichocarpa</i>			<i>P. tremula</i>	<i>P. trichocarpa</i>				
GAPDH	18	8	2	8	0	9.88600	0.0119393	0.01042
RPB1	10	2	2	5	1	4.39600	0.00615	0.00493
RPB2	12	2	6	4	0	5.04200	0.00803	0.00519
ITS	11	8	2	1	0	9.12500	0.01565	0.01531
TEF1- α	16	12	1	3	0	12.36100	0.01545	0.01503
Mean						8.16200	0.01144	0.01018
<i>P. balsamifera</i> vs <i>P. trichocarpa</i>			<i>P. balsamifera</i>	<i>P. trichocarpa</i>				
GAPDH	9	0	1	2	6	2.00000	0.00241	0.00001
RPB1	8	0	2	0	6	1.10800	0.00155	-0.00003
RPB2	7	0	3	1	3	1.142	0.00182	0.00016
ITS	2	0	1	0	1	0.12700	0.00022	0.00000
TEF1- α	4	0	1	1	2	0.70800	0.00089	0.00004
Mean						1.01700	0.00138	0.00004

^aFor analyses of the North American region, isolate TRTC150613 and isolates from Colorado from the unverified *Populus* host were included.

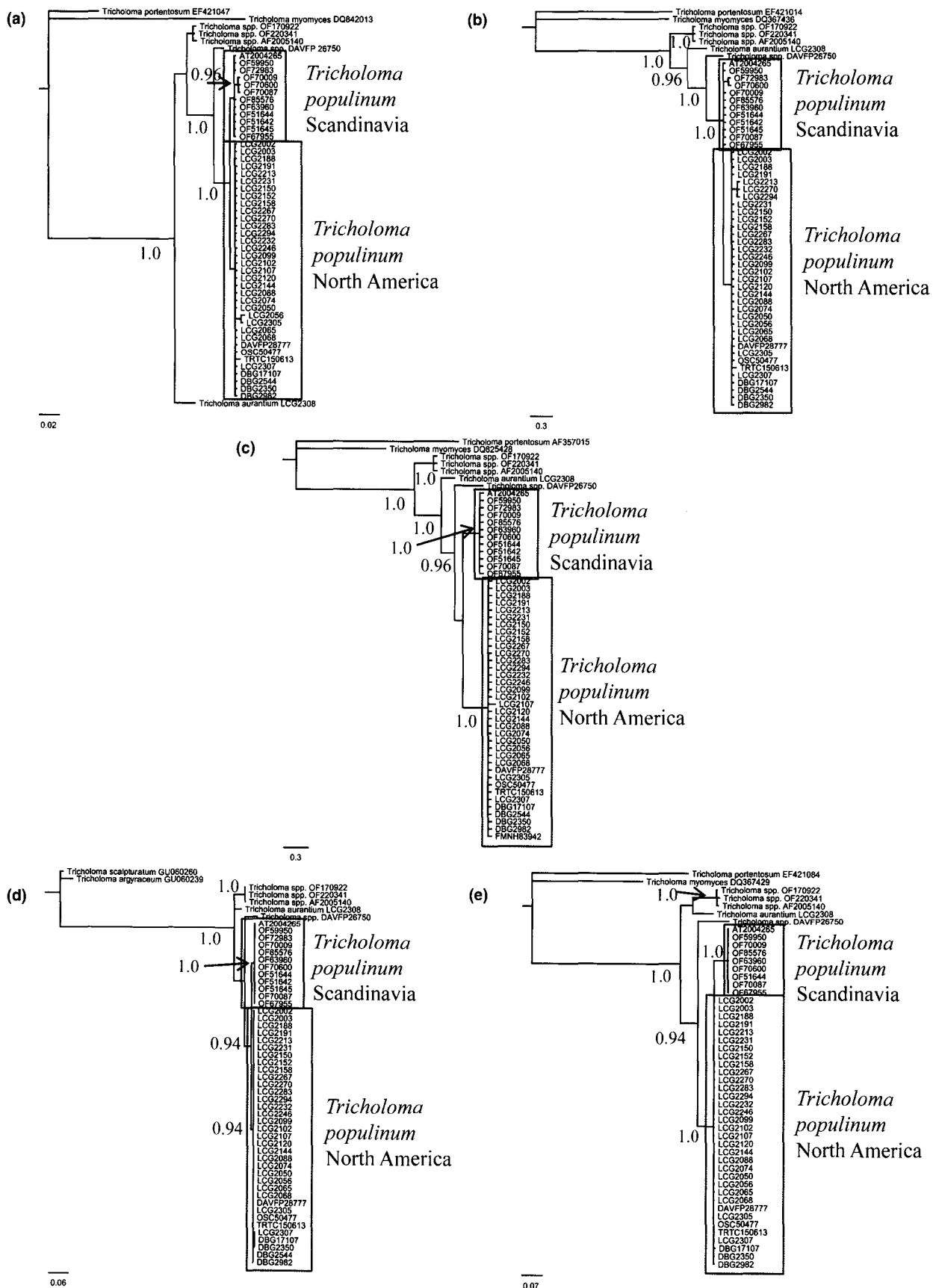
P1, mutations polymorphic in population 1, but monomorphic in population 2; P2, mutations polymorphic in population 2, but monomorphic in population 1; K, average number of nucleotide differences between populations; Dxy, nucleotide divergence (average number of nucleotide substitutions per site between populations); Da, number of net nucleotide substitutions per site between populations.

need to be sampled to assess population structure and host specificity across Europe. Populations of *T. populinum* from other *Populus* species, including quaking aspen, *P. tremuloides*, which is the North America sister species to *P. tremula*, are needed to provide a more complete North American phylogeographic history. Within North America the only potential population sampling of another *Populus* host came from samples from Colorado where *P. tremuloides* is dominant, although pockets of *P. balsamifera* are present. These isolates clearly group within the North American clade in each of the gene trees; however, without a clear identification of host species, no further conclusions are possible at this time.

Estimates of average genetic diversity were low but similar in both Scandinavia and North America (H_d , π , θ ; Table 2), reflecting recent demographic events. Low diversity is indicative of a recent founding event or postglacial recolonization bottlenecks (Hewitt, 2004), but also may reflect low mutation rate. Scandinavian populations of *P. tremula* survived in glacial refugia on the European continent (Birks *et al.*, 2008; Fussi *et al.*, 2010). In North America, recent studies of *P. balsamifera* found evidence for glacial refugia in southern central Canada (Keller *et al.*, 2010;

Levsen *et al.*, 2012) and possibly in a glacial-free region of Beringia (Hultén, 1937; Breen *et al.*, 2012) during the last glacial maximum c. 28 000–15 000 yr before present (Brubaker *et al.*, 2005). *T. populinum* may have survived with these hosts in glacial refugia and would then have undergone population contraction and expansion during glacial and interglacial periods.

While the number of biogeographic studies of ectomycorrhizal fungi is increasing (Douhan *et al.*, 2011), few Holarctic ectomycorrhizal fungi have well-documented phylogeographies. Studies are beginning to show a recurrent pattern of intercontinental divergence. The ectomycorrhizal fungi *Leccinum scabrum* and *Leccinum holopus* both have phylogeographic structure similar to *T. populinum*, and both *Leccinum* spp. have genetic discontinuities between the North American and European continents, and little intracontinental phylogeographic structure (den Bakker *et al.*, 2007). These *Leccinum* spp. are also host specialists, associated only with *Betula* spp., and occur only in the Northern Hemisphere (den Bakker *et al.*, 2007). In a recent study using microsatellites and nuclear loci, Vincenot *et al.* (2012) found the ectomycorrhizal fungus *Laccaria amethystina* formed phylogenetic lineages in Europe and Japan with no



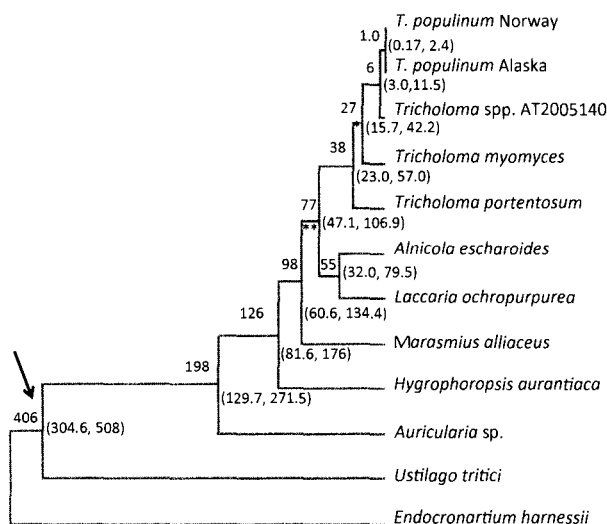


Fig. 3 Chronogram of select Basidiomycota based on BEAST analyses using a relaxed molecular clock. Numbers above branches are the estimated mean divergence dates in millions of years (Ma) based on a 430 ± 50 Ma calibration for the divergence of the Ustilaginomycotina and the Agaricomycotina, designated by the black arrow. Numbers in brackets at each node are the 95% highest posterior density (HPD), which encompasses 95% of the mean estimates for divergence time at a particular node from 15 000 trees. Posterior probabilities (PPs) were all = 1.0, except one had PP = 0.99 (**Agaricaceae/Tricholomataceae) and one had PP = 0.98 (**Tricholoma myomyces*/*Tricholoma* spp. and *T. populinum*).

shared haplotypes between regions. Genetic structure was not detected and only weak isolation by distance (IBD) was noted within Japan ($F_{ST} = 0.04$; 960 km between the two populations) and Europe ($F_{ST} = 0.041$; 2900 km between the most distance populations) (Vincenot *et al.*, 2012). Our results are very similar to those of Vincenot *et al.* (2012) at both inter- and intracontinental scales. We also found low differentiation of *T. populinum* populations in North America that were separated by as much as 2500 km (the maximum distance between our Pacific Northwest and interior Alaskan populations). A major difference between these study systems, however, is that *L. amethystine* associates with a variety of hosts, while *T. populinum* only occurs with *Populus* species. An interesting suggestion by Vincenot *et al.* (2012) is that *L. amethystine* may constitute a ring species. Future studies of Holarctic ectomycorrhizal fungi should also consider this hypothesis.

There are, however, also examples of intracontinental population structure within ectomycorrhizal species. A series of studies of *A. muscaria*, traditionally thought of as a host-generalist mycorrhizal fungus with a broad geographic range, have revealed strong allopatric divergence between Eurasia/Alaska and North

American lineages from similar habitats as a result of a lack of gene flow (Oda *et al.*, 2004; Geml *et al.*, 2006, 2008). However, in stark contrast to *T. populinum*, *A. muscaria* was demonstrated to be a phylogenetic species complex with strong divergence within North America attributed to ecoregional endemism (Geml *et al.*, 2006, 2008, 2010). Furthermore, phylogenetic species were found in sympatry in several regions (Geml *et al.*, 2006, 2008).

Tricholoma divergence time estimates

In this study, we estimated divergence time in *T. populinum* using two methods: one based on an estimated average nucleotide substitution rate for the ITS and the second based on a calibration point within the Basidiomycota but external to *Tricholoma*. Since the likelihood of discovering *Tricholoma* in the fossil record is very low, internal calibration of estimates of divergence time may never be possible. Hence we used calibration points between major groups within the Basidiomycota. It is clear that the lower substitution rate, 0.1×10^{-9} , is not appropriate, as these estimates of divergence for *Tricholoma* spp. from *T. populinum* (c. 150 Ma) are approximately the same as the estimated diversification of the Agaricales (Geml *et al.*, 2004; Matheny *et al.*, 2009). By contrast, the congruence of the estimates based on upper (0.87 Ma (95% HPD: 0.35, 1.46)) and average (1.7 Ma (95% HPD: 0.76, 2.95 Ma)) ITS nucleotide substitution rates and from calibration points (1.0 Ma (95% HPD: 0.17, 2.40)) within the Basidiomycota is striking. As sequences from more fungal genomes become available, robust estimates of nucleotide substitution rates for increasing numbers of representative Basidiomycota will be developed as well as development of numerous additional loci, both of which will increase the accuracy of divergence time estimates. It should be possible to refine the hypotheses presented here on the divergence time estimates among *T. populinum* lineages in future studies.

This was the first phylogenetic and phylogeographic study of *T. populinum*. The only other study examined genet size and longevity at a very fine spatial scale associated with *P. nigra* in France (Gryta *et al.*, 2006). Thus we know very little about the reproductive biology of *T. populinum*. The two *T. populinum* lineages identified here may be considered phylogenetic species, or cryptic species, according to genealogical concordance phylogenetic species recognition (GCPSR; Taylor *et al.*, 2000). The number of different lineages in the *T. populinum* complex can only be ascertained after additional sampling of hosts and geographic locations as discussed earlier. The degree to which *T. populinum* lineages remain interfertile and undergo hybridization as a result of secondary contact between North American *T. populinum* lineage(s)

Fig. 2 Bayesian consensus single gene phylogenies of *Tricholoma populinum* collections from North America and Scandinavia. (a) Phylogram based on *RPB1* locus (54 taxa, 742 characters). The DNA substitution model was GTR + invariant (I). (b) Phylogram based on *RPB2* locus (54 taxa, 628 characters). The DNA substitution model was GTR + gamma (Γ). (c) Phylogram based on internal transcribed spacer (ITS) sequences (55 taxa, 604 characters). The DNA substitution model was GTR + Γ . (d) Phylogram based on *GAPDH* locus (54 taxa, 885 characters). The DNA substitution model was GTR + Γ . (e) Phylogram based on *TEF1- α* locus (51 taxa, 801 characters). The DNA substitution model was GTR + Γ . Posterior probabilities > 0.95 are shown. *Tricholoma myomyces* and *Tricholoma portentosum* were used as outgroups. Closely related herbarium collections that were identified as *T. populinum* were included in order to assess their phylogenetic relationship across several loci (DAVFP26750, OF170922, OF220341, AT2005140). *Tricholoma aurantium* LCG2307 is a closely related species.

and *T. populinum* lineage(s) associated with European or Asian poplars has not been examined. In this study, North American *T. populinum* populations from field collections were associated with host populations devoid of introduced *Populus* spp. *Populus* spp. frequently hybridize and it may be possible to examine hybridization of *T. populinum* from known *Populus* hybrid zones in future work.

Our results provide strong evidence for a lack of ongoing gene flow between North American and Scandinavian populations of *T. populinum*. The emerging trend of intercontinental genetic breaks in ectomycorrhizal fungi from mid-latitudes implies that fungal biogeography will be a rich area of inquiry, with relevance to past and present organismal responses to climate change.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Results from Structure analyses.

Fig. S2 Results from *Beast for *Tricholoma*.

Fig. S3 Results from *Beast for *Populus*.

Table S1 Isolates used in this study and associated GenBank accession numbers

Table S2 Misidentified isolates used in this study and associated GenBank accession numbers from BLAST

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